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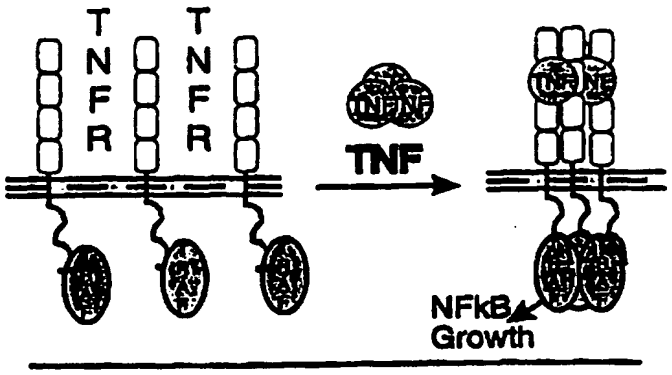

(57) Abstract

Compounds and methods for interrupting the interaction between Epstein-Barr virus encoded proteins known as LMP1 and Tumor Necrosis Factor Receptor Associated Factors (TRAFs), particularly novel human TRAFs, thereby inhibiting lymphoblast growth and tumorigenesis, particularly Hodgkin's disease, Burkitt's lymphoma, lymphomas seen in immunocompromised patients (including AIDS-associated central nervous system lymphomas), and nasopharyngeal carcinomas. Therapies for treating EBV infection are also disclosed, e.g. in patients with infectious mononucleosis, by blocking the establishment of latent infection and/or blocking lytic infection. Compounds and methods for controlling TRAF-Mediated TNF/TNFR signaling by administering to a TRAF-encoding cell a compound that inhibits TRAF oligomerization are also disclosed. Compounds and methods for controlling cell growth and death based on the interaction of TNF receptor family carboxy terminal cytoplasmic domains with human TRAFs, e.g., LAP1 and EBI6. These interactions are particularly important in controlling cells in the immune system and regulating immune responses. They are also important for controlling abnormally growing cells, that is cancer cells.

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CONTROLLING TRAF-MEDIATED SIGNALS

Field of the Invention

This invention is in the general field of compounds (including proteins) and methods for controlling cell growth (or tumorigenesis), particularly cell growth/tumorigenesis related to viral infections, such as Epstein-Barr virus (EBV) infection. The invention also relates to therapeutic strategies for treating viral infections and conditions characterized by abnormal or undesired immune functions.

Background of the Invention

I. Epstein-Barr Virus LMP1

EBV is a herpesvirus that infects B lymphocytes and certain epithelial cells. EBV causes lymphoproliferative diseases and tumorigenicity in humans, which manifest particularly as infectious mononucleosis, Hodgkin's disease, Burkitt's lymphoma, lymphomas seen in immunocompromised patients (including AIDS-associated central nervous system lymphomas), and nasopharyngeal carcinomas, the latter being particularly prevalent in populations of southern Chinese extraction. See, Kieff and Liebowitz, "Epstein-Barr virus and its replication" *Virology*, B.N. Fields and D.M. Knipe, eds., New York Raven Press, pp. 1889-1920 (1990); and Miller, "Epstein-Barr Virus" *Virology*, B.N. Fields and D.M. Knipe, eds., New York Raven Press, pp. 1921-1958 (1990).

An EBV-encoded protein termed latent infection membrane protein 1 (LMP1) induces most of the phenotypic effects of EBV B-lymphocyte infection. LMP1 has been characterized as an integral membrane protein which consists of a 23 amino acid amino terminal cytoplasmic domain, six markedly hydrophobic transmembrane domains separated by short reverse turns, and a 200 amino acid

carboxy terminal cytoplasmic domain (Fennewald, et al., *J. Virol.* 51:411-419 (1984); and Hennessy, et al., *Proc. Natl. Acad. Sci. USA* 81:7201-7211 (1984)). The transmembrane domains enable LMP1 to post translationally insert into membranes and to accumulate in aggregates in the plasma membrane (Hennessy, et al., *Proc. Natl. Acad. Sci. USA* 81:7201-7211 (1984); Liebowitz, et al., *J. Virol.* 58:233-237 (1986).

LMP1 has been implicated as essential for B-lymphocyte transformation (Kaye et al., *Proc. Natl. Acad. Sci. USA* 90:9150-9154 (1993)). From the observation that LMP1 forms patches in the plasma membrane, Wang et al., (1985) *Cell*, 43:831-840 suggest that it could be part of a complex that might play a direct role in virus induction of cell proliferation. Based on LMP1's capacity to transform established cells, Wang et al. conclude that LMP1 mimics the cell growth effect of tyrosine kinase oncogenes and *ras* genes. In so doing, Wang et al. point out that LMP1 is not significantly homologous to any of those oncogene products, and they conclude that it may affect cell growth by a different mechanism. They further conclude that LMP1 is unlikely to be a receptor for a growth factor and, by analogy to other oncogenic events which frequently involve more than one gene, they conclude that EBV nuclear protein genes are likely to be necessary complements to LM1P in the growth transformation of primary B cells. Finally, they speculate that patching could be important in the biologic effect of LMP1, because: a) LMP could interact with a growth factor receptor; and b) growth factors are essential to the continued proliferation of EBV transformed lymphocytes. Kaye et al., *Proc. Natl. Acad. Sci. USA* 90:9150-9154 (1993) also indicate that LMP1 transmembrane domains are important for conferring plasma membrane aggregation.

II. TNF/TNFR Signaling

Tumor necrosis factor (TNF) is a cytokine produced mainly by activated macrophages, which elicits a wide range of biological effects related to endotoxic shock, inflammatory, immunoregulatory, proliferative, cytotoxic, and anti-viral activities. See generally Tartaglia and Goeddel (1992) *Immunology Today* 13:151-153; Goeddel et al. (1986) *Cold Spring Harbor Symp. Quant. Biol.* 51:597-609; Beutler and Cerami (1988) *Ann. Rev. Biochem.* 57:505-518; and Fiers (1991) *FEBS Lett.* 285:199-212. The various cellular responses mediated by TNF are initiated by its interaction with two distinct cell surface receptors of approximately 55kDA (TNF-R1) and 75kDa (TNF-R2). See Tartaglia and Goeddel, cited above; and Rothe et al. (1992) *Immunol. Res.* 11:81-90. These receptors are also known, respectively as p60TNFR and p80TNFR. The independent signaling responses mediated by these two receptors have been studied. See Rothe et al. (1994) *Cell* 78:681-692 and publications cited therein. Both receptors are members of the larger TNF receptor superfamily having certain common structural and functional characteristics. Other TNFR family members include CD30, CD27, CD40, lymphotoxin-β receptor, OX-40, 4-1BB, and CD95 (Fas). See generally Smith et al. (1994) *Cell*, 76:959-962; and Beutler and van Huffel (1994) *Science*, 264:667-668.

TNF-receptor superfamily signaling (including NFκB signaling) is involved in cell growth and cell death. Such signaling appears to require the aggregation of receptor monomers in a process initiated by ligand binding. The multimerization of receptor cytoplasmic domains is postulated to unveil discrete chain motifs or to create composite tertiary epitopes that attract or activate constitutively associated molecules that are components of intracellular signal transduction pathways.

See, Bazan (1993); and Tartaglia et al. (1993b). In general, notwithstanding attempts to implicate second messenger systems in TNF signaling, the mechanism for coupling of second messenger systems to TNF receptors remains an enigma. See, Pfizenmaier et al. (1992); Kolesnick and Golde (1994); and other articles cited in Rothe et al. (1994), cited above.

Rothe et al. (1994) report cloning of two murine signal transducers associated with the cytoplasmic domain of a member of the TNF receptor superfamily (TNF-R2). They designate those transducers TRAF1 and TRAF2. They report that TRAF1 and TRAF2 form a heterodimeric complex associated with the cytoplasmic domain of murine TNF-R2 (see Figure 8 of Rothe et al.). They conclude:

> "By virtue of their ability to associate with the cytoplasmic domain of TNF-R2, TRAF1 and TRAF2 define a novel class of putative signal transducers. However, their functional roles and mechanisms of action in TNF-R2-mediated signaling are presently unresolved. Of particular interest will also be their mode of action after binding of the ligand TNF to the receptor."

Summary of the Invention

There are several aspects of the invention separately summarized below.

I. Inhibiting LMP1-LAP1 Interaction

We have discovered a novel B-cell protein which we term "LMP1 Associated Protein 1 or LAP1", which strongly associates with a cytoplasmic carboxy terminal domain of EBV LMP1. This LMP1 domain is an essential component of LMP1 for effecting cell growth transformation. LAP1 is related to murine TRAF2. We have also discovered a related novel B cell protein induced by EBV infection, which we term Epstein-Barr Induced protein 6 or EBI6.

EBI6 has extensive homology to murine TRAF1 and appears to be its human homolog.

Our findings are that: a) LMP1 expression clusters LAP1 and EBI6 to lymphoblast plasma membrane patches; b) LMP1 co-immunoprecipitates with LAP1 or EBI6; c) LAP1 can directly and biochemically interact with LMP1, particularly with a 44 amino acid segment of the LMP1 carboxy terminal cytoplasmic domain that is stringently required for transformed cell growth; d) LAP1 directly and biochemically interacts with the cytoplasmic domains of p80TNFR, CD40 and the lymphotoxin-β receptor; e) to a lesser extent, LAP1 interacts with the cytoplasmic domains of p60TNFR and Fas; f) EBI6 associates strongly with p80 TNFR and, to a lesser extent, with p60TNFR.

We conclude that the lymphoblast growth and tumorigenesis effects of EBV depend on LMP1-associated TNFR-signal transduction, and that the TRAFs represent a necessary link in such EBV-induced signal transduction. Specifically, we conclude that TRAF oligomerization effected by LMP1 interaction is a necessary step in EBV-induced lymphoblast growth and tumorigenesis. See Figure 6B.

A particularly functional use for our discovery involves new screening techniques, compounds and methods for interrupting the LMP1-related, TRAF-mediated signaling and thus inhibiting lymphoblast growth and tumorigenesis. This use of the invention applies generally to virally induced tumors (particularly but not exclusively of lymphoid cells). The use particularly applies to Hodgkin's disease, Burkitt's lymphoma, lymphomas seen in immunocompromised patients (including AIDS-associated central nervous system lymphomas), and nasopharyngeal carcinomas. The invention also provides therapies for treating viral infection, e.g. in EBV-infected patients with infectious mononucleosis, by

blocking the establishment of latent infection and/or blocking lytic infection. While we postulate a specific molecular mechanism, the effectiveness of the therapeutic compounds and methods described herein and the practice of the invention do not depend on the completeness or accuracy of (and we do not wish to bind ourselves to) any particular theory.

Accordingly, one aspect of the invention features methods of treating infection and controlling cell growth/tumorigenesis associated with a LMP1-encoding viruses (particularly EBV), by administering to infected cells a compound that inhibits TRAF-mediated virally induced TNFR cell growth, cell death, and/or NFκB signaling. It particularly features inhibiting interaction between EBV LMP1 and a Tumor necrosis factor Receptor Associated Factor (TRAF).

Two categories of compounds that inhibit LMP1-TRAF interaction are: A) polypeptides that interact with LMP1, particularly with a 44-amino acid TRAF-interacting domain of LMP1 (amino acids 188-231) discussed below;[1] and B) polypeptides that interact with TRAF proteins at a LMP1-interacting TRAF domain, at a TRAF-TRAF interacting domain that is necessary for TRAF oligomerization (or TRAF-TRAF interaction), or at a TNFR-interacting TRAF domain.

A. LMP1-1 Interacting Inhibitors

Examples of category A above (LMP1-interacting inhibitors) are polypeptides that include a LMP1-interacting TRAF domain, particularly a LMP1-interacting domain of a human TRAF such as a LAP (most preferably LAP1). The TRAF domain of the inhibitors may be a LMP1-

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[1] The sequence (SEQ ID NO: 3) is a 44 amino acid sequence at the beginning of the C-terminal cytoplasmic domain; see Fennewald et al. *J. Virol.*, 51:411-419 (1984), hereby incorporated by reference: G Q R H S D E H H H D D S L P H P Q Q A T D D S G H E S D S N S N E G R H H L L V S G A.

binding TRAF epitope contained on the C-terminal side of the TRAF coiled coil (Leu zipper) domain described below, for example an epitope on the LAP1 segment represented by the hybrid protein described below, G4TADLAP1 (amino acids 346-568 of SEQ ID NO:1). Those skilled in the art will readily recognize that specific LMP1-interacting LAP1 sequences can be identified using standard techniques by making proteolytic (e.g., V8 enzyme or trypsin) or recombinantly produced fragments of the above LAP1 segment (aa 346-568). The resulting fragments are then tested for interaction using any of the screens described elsewhere in this application. One LAP1 sequence of interest includes amino acids 346-368 of SEQ ID NO:1 -- E A D S M K S S V E S L Q N R V T E L E S V D. A second LAP1 sequence of interest is the C-terminal sequence (amino acids 407-568 of SEQ ID NO:1). If the LMP1-interacting domain is not contained therein, these sequences can be lengthened in an iterative process or other fragments may be screened until a minimal LMP1-interacting LAP1 sequence is identified.

B. TRAF-protein-interacting Inhibitors

One class of category B (TRAF-interacting) inhibitors are polypeptides that include a LMP1 sequence (particularly the above-described 44-amino acid sequence, SEQ ID NO: 3)) that interacts with a TRAF protein such as a human LAP, particularly human LAP1, and can therefore prevent the TRAF protein from interacting with LMP1. A second class of TRAF-interacting inhibitors are polypeptides that include a TRAF (e.g., a human TRAF such as EBI6 or a human LAP such as LAP1) oligomer-forming coiled coil domain -- i.e., a TRAF domain involved in formation of TRAF-TRAF hetero- or homo-oligomers. Such domains can be recognized by a repeating pattern in which every seventh (more or less) amino acid residue is Leu,

Ile, or Val. See generally, Figure 1. One specific such domain includes the LAP1 sequence from amino acids 309-341 of SEQ ID NO:1, inclusive: L R N N E S K I L H L Q R V I D S Q A E K L K E L D K E I R P F R. A second specific such domain includes the EBI6 sequence from amino acids 194-224 of SEQ ID NO:2, inclusive: L R V F E N I V A V L N K E V E A S H L A L A T S I H Q S Q L. Either of the above domains can be extended, for example, to include the LAP1 sequence between amino acids 263-400 or EBI6 amino acids 187-257. As described above with respect to LMP1-LAP1 interaction, oligomerizing (or TRAF-interacting) LAP1 or EBI6 fragments can be identified using standard fragmentation and screening techniques, in which the above fragments, or longer polypeptides containing all or a significant part of them, are tested for TRAF interaction.

A third class TRAF interacting inhibitors includes inhibitors that have a TNFR (e.g., TNFR p60 or p80) TRAF-interacting domain. This third class of inhibitors is used primarily in the second aspect of the invention (II., below), and so we will describe it later with respect to that aspect of the invention. Nevertheless, it is possible that these inhibitors will also inhibit LMP1-TRAF interaction.

This first aspect of the invention specifically includes the polypeptide inhibitors described in the above methods and recombinant DNA encoding those inhibitors. Preferably, the recombinant nucleic acid further comprises regulatory DNA positioned to transcribe the polypeptide encoding DNA. The recombinant DNA itself may be formulated for use in gene therapy, --i.e., for administration to the patient as DNA expressed in the patient to produce the therapeutic polypeptide, or it may be used as a reagent to produce therapeutic polypeptides

in conventional fermentation processes. In either case, the invention may also feature cells comprising the above recombinant nucleic acid, including the regulatory DNA required to express the polypeptide. The invention also includes methods of making the purified polypeptide by culturing a cell comprising the recombinant nucleic acid and recovering the purified polypeptide from the cells or the culture medium. The method also includes recombinant polypeptides and antibodies thereto -- i.e., antibodies raised against, or antibodies that specifically bind to, the polypeptides.

This aspect of the invention is particularly useful for treating EBV-infected patients described above. Details of the invention will be apparent from the description below of TRAFs and of methods that establish and evaluate inhibition of the LMP1-TRAF interaction.

This aspect of the invention also features one of the above-described TRAF signal-effecting inhibitors formulated for administration as a therapeutic. Further, it features polypeptides that have been modified as described in greater detail below, while preserving the inhibitory effect of the native sequence.

This aspect of the invention also features procedures for screening candidate compounds for their ability to inhibit LMP1-TRAF or TRAF-TRAF interaction. *In vitro* and *in vivo* screens are described in greater detail below.

The peptides of the invention may also be used as specialty chemicals to control growth of EBV-transformed cell cultures.

II. Controlling TRAF-Mediated TNF/TNFR Family Cell Growth/Death Signaling

A second major aspect of the invention features controlling TRAF-mediated cell growth/death signaling via the TNFR superfamily of receptors. As described above, this family includes a number of receptors characterized by common functional and structural features. For a general review of TNFR's, see Smith et al., *Cell* 76:959-962, (1994) hereby incorporated by reference). The invention includes controlling TNFR cell growth/death signals that are a response to direct interaction with the receptor ligand, or (as described above) a response to interaction with other macromolecular species such as LMP1. It also includes controlling signals that result from mutations in receptors that lead to abnormal cell growth/death signaling. We specifically include (without limitation) signaling via TNFR family members such as p80, CD40, and lymphotoxin-β receptor that interact directly with LAP1. Less preferred instances of TNFR signaling controlled by the invention include p60 and Fas signaling.

Specifically, our discovery indicates that TRAFs are an important component in TNF-TNFR cell growth/cell death or NFκB signaling. Indeed, LAP1 interacts directly with p80, LTβ, CD40, and (to a lesser extent) with p60 and Fas, and such interaction is an essential step in the signal pathway. Thus, the second aspect of the invention features controlling TRAF-mediated TNFR cell growth/death signals independent of EBV or other viral infection.

In this aspect of the invention TRAF-Mediated TNF/TNFR signaling is controlled by administering to a TRAF-encoding cell a compound that interacts with a TRAF or with a TNFR to inhibit signaling function.

Preferred compounds are those that interact with a TRAF, for example, with an oligomerizing TRAF domain,

particularly an oligomerizing domain of a human TRAF. Inhibitors according to this aspect of the invention include polypeptides having a coiled coil TRAF domain. One such human TRAF domain is a human EBI6 coiled coil domain, for example, the EBI6 sequences described above. Human LAP (particularly LAP1) coiled coil domains described above are also included. Inhibitors containing the coiled coil domain of LAP1 or (more preferably) EBI6 described below will competitively bind to TRAFs without effecting a signal because such binding will inhibit TRAF oligomerization and thereby inhibit the TNF-TNFR growth signal pathway.

A second class of TRAF-mediated signaling inhibitors are those that directly involve other TRAF domains, such as the C-terminal TNFR-interacting LAP1 domain contained in amino acids 407-568 of LAP1 (SEQ ID NO:1) or the EBI6 domain contained in amino acids 259-416 of EBI6 (SEQ ID NO:2). The metal binding domains (see the RING finger motif of LAP1 and the zinc finger structure of EBI6 that are underlined in Figure 1) are likely to chelate to metal ions and function as mediators of macromolecular interactions in the TNFR cell growth/death signaling. Thus, these metal binding domains are also candidate inhibitors of such signaling.

Administration of these inhibitors is indicated for patients with undesired lymphocyte proliferation, particularly autoimmune diseases such as rheumatoid arthritis, Crone's disease, lupus, or patients requiring immunosuppression for organ transplantation.

As above, this aspect of the invention includes not only polypeptide inhibitors and effectors, but also the DNA encoding them and cells containing that DNA, for use as a therapeutic or as biological materials or as reagents in a method for making them. It also includes recombinant polypeptides and antibodies to the

polypeptides. Similarly, this aspect of the invention features *in vivo* and *in vitro* screens for identifying compounds that inhibit TRAF oligomerization and that mimic the TRAF TNF-TNFR signal-mediation function.

This aspect of the invention particularly includes treatments of patients with non-EBV-associated Hodgkin's disease. It is interesting to note that high CD30 level found in Hodgkin's disease may result from faulty TRAF, acting as an oncogene to disrupt the TNFR family-mediated death signals or their cell growth effects. This aspect of the invention also includes treatment and diagnosis of patients characterized by a TRAF mutation.

Enhanced cell control of the cell death signal might be desirable in treatment of other tumors. For example, some viruses may disrupt the immune response by disrupting the normal TNFR cell growth/death signal (e.g., by secreting ineffectual TNF-competitors that prevent TNFR activation). Others may disrupt the cell death signal by acting downstream at a TRAF-mediated portion of the signal pathway. In either event, such viruses may be treated by the addition of TRAFs or TRAF domains that are effective to mediate the cell death signal, notwithstanding the viral intervention. Alternatively, compounds identified in the screens below as augmenting the cell death signal could be used.

Thus, this aspect of the invention may have application to virally induced neoplasms that are not characterized by a LMP1-type molecule, such as hepatitis virus-induced hepatocarcinoma, human papilloma virus-induced cervical cancer, and adult T-cell leukemia induced by HTLV-I.

Finally, control of TRAF-mediated cell growth/death may be useful as a generalized transient immune enhancer (adjuvant) to accompany a specific

vaccine for inducing immunity to infectious agents or abnormal cells.

This aspect of the invention also features enhancing TNF/TNFR cell growth/death signals by providing additional TRAF to mediate those signals. We have discovered two specific proteins useful in such signal enhancement, which are Human LMP1-Associated Protein (LAP1) and Human Epstein-Barr virus Induced Protein-6 (EBI6).

Other embodiments are within the following claims.

Description of the Preferred Embodiments

As summarized above, one aspect of the invention features inhibiting interaction between a viral LMP1 and a TRAF. Figure 6B is a diagram of this interaction, which ultimately results in NFκB mediated growth signaling. At the outset, we emphasize that our invention extends beyond the narrow bounds of the experiments described in detail below. It specifically extends beyond EBV and LAP1.

A second aspect of the invention focuses on TRAF-mediated signaling that does not require LMP1, as illustrated by Figure 6A. In this aspect, such signaling is inhibited as described above.

I. The Viruses Being Inhibited

While EBV is the only herpesvirus currently known to encode a LMP1 protein and our discussion of this aspect of the invention concentrates on treatment of EBV-infected patients, our discoveries concerning the mechanism of EBV-induced cell growth and tumorigenesis can be readily applied to other viruses that induce cell growth and/or tumors or that block cell death through a TNFR family member-interacting TRAF. The target viruses also preferably (but not necessarily) will encode a membrane protein having comparable structure and

function; alternatively, the virus may encode a cytoplasmic protein that aggregates to activate TRAFs.

Structurally, LMP1 proteins are viral proteins that include N- and C-terminal cytoplasmic domains, connected by multiple intervening hydrophobic transmembrane domains separated by short reverse turns. These transmembrane domains enable the protein to post translationally insert into membranes and to accumulate in aggregates (oligomers) in the plasma membrane. Functionally, LMP1s have broad transforming effects as membrane aggregated proteins. Presumably they activate a growth factor receptor pathway.

Accordingly, our invention broadly features inhibiting interaction with LMP1 proteins, and we do not limit ourselves in this aspect of the invention to EBV therapies. Preferably, the invention features EBV-related therapies, including therapies for infection with any EBV strain or type.

II. Characteristics Of TRAF Mediators Being Inhibited

As discussed above, our discovery relates to specific signaling pathways mediated by TRAFs. We use the term TRAF to describe the family of signal transducing proteins that mediate TNF/TNFR cell growth/death and NFκB signals. TRAFs are characterized by association with the cytoplasmic domain of a Tumor Necrosis Factor Receptor. The TRAFs of interest in this aspect of the invention can be identified by the following characteristics.

First, TRAFs include a C-terminal intracellular domain that begins with an extended coiled coil motif. The coil motifs of different TRAF molecules could be involved in oligomerization of these molecules and generation of complexes involved in signaling. Some, but not all, TRAFs interact strongly with LMP1 proteins (described above). For example, they interact with the

above-described 44 amino acid LMP1 domain implicated in B cell growth and tumorigenesis. TRAFs are generally capable of interacting with the cytoplasmic domains of multiple TNFR's as described above (e.g., the 78 C-terminal amino acids of TNF-R2). It is possible that the same TRAF domain that interacts with LMP1 also interacts with the cytoplasmic domain of a TNFR. Some, but not all, TRAFs contain an N-terminal RING finger sequence motif -- a cysteine rich protein motif related to other zinc finger motifs involved in protein-DNA and protein-RNA interaction. Methods and procedures for identifying further TRAFs will be apparent from the following description of our discovery of LAP1 and EBI6 and from the above description of screening methods.

We have specifically identified two human TRAFs and confirmed their direct biochemical association with the various members of the TNF receptor family. Thus, LAP1 interacts directly with the cytoplasmic domain of TNFR p80, LTβ, CD40, and (to a lesser extent) with p60 and Fas. A second novel human TRAF, EBI6 interacts directly with the p80 cytoplasmic domain and (to a lesser extent) with the p60 cytoplasmic domain. Also, in the case of LAP1, we also have confirmed interaction with LMP1.

The TRAF domain may be defined on the basis of maximal (>50%) collinear primary sequence identity with the carboxy terminal 230 amino acids of a known TRAF, such as the murine TNF receptor associated proteins TRAF1 and 2. Genetic and biochemical data link TRAF1 and 2 to the cell growth, cell death, and NF-κB transducing effects of a domain near the carboxy terminus of the p80 TNF receptor cytoplasmic tail (Rothe, et al., *Cell* 78:681-692 (1994)).

The sequence of LAP1 and EBI6 is compared to TRAF1 and 2 in Figure 1. Specifically, Figure 1 depicts

alignment of the amino acid sequences of human EBI6 and LAP1 and murine TRAF1 and TRAF2 using the CLUSTAL program (PCGene; IntelliGenetics). Identical (stars) and homologous (dots) amino acids are shown. Pairwise alignment of EBI6 and TRAF1 is also shown with identical amino acids designated by bold faced characters. Amino acids that form the RING finger motif in LAP1 and TRAF2 and the Zn finger structure in EBI6 and TRAF1 are underlined. Amino acids that form putative coiled coil structures are boxed. The TRAF domain is shown by large boxes.

EBI6 is almost certainly the human homolog of murine TRAF1 based on collinear 86% primary sequence identity, the expression of both in lung but not in most other tissues, and the amino terminal zinc finger motif. LAP1 is not the human homolog of murine TRAF2, but rather the existence of these two molecules is indicative of a larger repertoire of ring finger TRAFs. LAP1 is similar to TRAF 2 in size, in having an amino terminal RING finger domain, and in being constitutively expressed in most tissues. However, while LAP1 is 45% identical to both TRAF 2 and TRAF 1 in the TRAF domain, LAP1 is quite divergent from TRAF2 outside of the TRAF domain and is only 27% identical to TRAF 2 overall. LAP1 also appears to differ from TRAF2 in not interacting with EBI6, the human homolog of TRAF1.

As demonstrated by the examples below, LAP1 and EBI6 interact with the cytoplasmic domain of not only the p80 TNF receptor but also p60, albeit less strongly than with p80. This is the first evidence of interaction beyond p80 between these putative effectors and the TNF receptor family. Indeed, LAP1 can also interact strongly with the CD40 cytoplasmic domain as well as the lymphotoxin-β receptor, and, to a lesser extent, with Fas cytoplasmic domain (Figure 5B).

We have identified human TRAFs in the context of an investigation into the mechanisms by which LMP1 transforms cells, and a significant result of these experiments is the establishment of a connection between LMP1 and TNF receptor signaling pathways. LMP1 binds directly to LAP1 and also interacts with EBI6 in human lymphoblasts. Our genetic, biochemical and intracellular localization data on the interaction of LMP1 with LAP1 and EBI6 reinforce the previous genetic and biochemical linkage of murine TRAF 1 and 2 to the TNF receptor cytoplasmic domains in supporting a role for the TRAFs as mediators of growth/death and NF-κB responses.

Figures 6A and 6B are schematic models of TRAF mediated signal transduction. Figure 6A shows p80 TNF receptor activation as described above and Figure 6B shows LMP1 complexes (Figure 6B) at the plasma membrane. A model for the activation of the p80 TNF receptor is shown in Figure 6A. The extracellular region of the TNF receptor is composed of four domains with characteristic cysteine patterns. The cytoplasmic domain of the receptor is known to associate with TRAF molecules (TRAF). Upon binding of TNF (shown here as a trimer) the extracellular domains of several receptor molecules are believed to be crosslinked causing aggregation of intracellular domains and their associated TRAF molecules. Clustering of receptor molecules and their intracellular domains results in signal transduction as manifested by a number of phenotypic alterations including the activation of the transcription factor NF-κB and cell growth or death signaling.

In Figure 6B three LMP1 molecules are shown to form a constitutive complex at the plasma membrane (depicted by the gray area between the two solid horizontal lines). The amino terminal (N) and carboxy terminal (C) cytoplasmic regions of LMP1 are shown by

short and long lines respectively. The transmembrane domains of LMP1 are depicted by vertical cylinders which are joined by short reverse turns (short curved lines). Aggregation of LMP1 molecules at the plasma membrane brings together LMP1 associated TRAF molecules (TRAF) in a complex thus generating a constitutive signal that results in pleiotropic effects including activation of NF-κB cell growth signaling.

Additional TRAF proteins are likely to emerge with screening, generally as described below in Example I for LAP1 and EBI6. LAP1 was identified twice in a our yeast two hybrid screen of $5X10^5$ cDNAs and no other strong interaction was identified, providing a direct and possibly exclusive linkage between LMP1 and LAP1. In yeast, the interaction of LAP1 with LMP1 is through the membrane proximal 44 amino acids of LMP1 and the last 223 residues that comprise the LAP1 TRAF domain. The interaction with the first 44 amino acids of the LMP1 carboxy terminus is significant since EBV recombinants deleted for this region apparently do not initiate fibroblast independent growth of primary human B lymphocytes, whereas an EBV recombinant which expresses LMP1 with only the first 44 amino acids of the carboxy terminal cytoplasmic domain can be grown on fibroblasts. The interaction between LMP1 and LAP1 is likely to be mediated by hydrophilic residues of the TRAF domain since the first 44 amino acids of the LMP1 carboxy terminal domain are remarkably hydrophilic. The induction of EBI6 by latent EBV infection is also consistent with an important role of EBI6 in EBV-mediated growth transformation of B lymphocytes.

The six markedly hydrophobic transmembrane domains of LMP1 enable it to aggregate in the plasma membrane and to present aggregated cytoplasmic domains to the TRAFs (Figure 6B). In presenting aggregated TRAF interacting

domains, LMP1 mimics TNF-receptor aggregation which appears to be essential for signal transduction (Engelmann, et al., 1990; Loetscher, et al., 1991; Pennica, et al., 1992; Tartaglia and Goeddel, 1992). Receptor crosslinking or LMP1 expression probably bring TRAF and associated molecules in close proximity creating a second messenger signal perhaps mediated by the receptor associated serine threonine kinases (Darnay, et al., 1994a; Darnay, et al., 1994b; VanArsdale and Ware, 1994). Since LMP1 constitutively aggregates LAP1 and EBI6 in oligomeric complexes at plasma membrane patches these complexes could constitutively activate growth signals and NF-kB in the absence of extracellular stimuli. LMP1 signaling via TRAF molecules thus may proceed independently of TNF receptor molecules. Alternatively, LMP1 may stabilize the interaction of TNF family receptor-TRAF aggregates in constitutively active plasma membrane complexes. In fact, some evidence favors the latter alternative in that lymphotoxin-α (TNFβ) is an autocrine growth factor for EBV-transformed lymphoblastoid cell lines (Estrov, et al., 1993; Gibbons, et al., 1994). Further, expression of the full range of EBV latent infection associated proteins in Burkitt' lymphoma cell lines induces TNFβ and the p80 receptor (Gibbons, et al., 1994). Moreover, antagonistic antibodies to the p60 TNF receptor have a negative growth effect in such cells (Gibbons, et al., 1994). The LMP1 cytoplasmic carboxy terminal domain and TNFR family members could even interact with different domains of the same LAP1 molecule since there is no obvious homology between the LMP1 cytoplasmic carboxy terminus and the cytoplasmic domains of TNFR family members.

The induction of EBI6 by latent EBV infection and the association of EBI6 with LMP1 in B lymphoblasts are also evidence of an important role for EBI6 in EBV

mediated B lymphocyte growth transformation. The interaction appears to be less direct than with LAP1 and may be mediated by another as yet unidentified human RING finger TRAF.

TNFα and CD40 ligand are well known mediators of growth of B lymphocytes and of other cell types that are targets for LMP1 transforming effects (Noelle, et al., 1992; Boussiotis, et al., 1994). In fact CD40 ligation and IL4 treatment are sufficient to sustain the proliferation of primary B lymphocytes in vitro for several months and the cells are phenotypically similar to EBV transformed lymphocytes (Saeland, et al., 1993; Banchereau, et al., 1994; Galibert, et al., 1994). The LTβR is expressed on epithelial cells; while basal epithelial cells and anaplastic nasopharyngeal carcinoma (NPCs) cells also express high levels of CD40 (Busson et al., 1988; Young, et al. 1989). LMP1 through constitutive direct interaction with LAP1, may amplify or usurp LTβR and CD40 signal transduction and constitutively promote cell growth. NPC is tightly associated with EBV and LMP1 is frequently expressed in the tumor cells (Brooks, et al., 1992). Hodgkin's disease is another EBV associated malignancy in which LMP1 is expressed (Herbst, et al., 1991). CD40, TNF receptors and the related CD30 receptor are up regulated in Hodgkin's disease cells (Froese, et al., 1987; Pfreundschuh, et al., 1989; Carde, et al., 1990; O'Grady, et al., 1994; Trumper, et al., 1994). Therefore a potentially important consequence of the demonstrated interaction between LAP1 and LMP1 is that inhibitors of that interaction may affect the growth or development of these LMP1 associated malignancies.

In interacting with components of receptor signaling, LMP1 is somewhat similar to BPV E5 which dimerizes in the plasma membrane, presumably through

hydrophobic interactions, and activates receptors for EGF, PDGF, or CSF-1 (Martin, et al., 1989; Petti, et al., 1991; Petti and DiMaio, 1992). E5 binds a component of vacuolar H^+-ATPases and this may affect receptor recycling (Goldstein, et al., 1991).

We found that LAP1 or EBI6 localize to vesicle like structures in the cytoplasm and are localized to the plasma membrane by LMP1 expression in the same cells. See, Figures 4A-4P, described below. Further, LMP1 localizes EBI6 to the plasma membrane despite its inability to directly interact with EBI6, indicating that there is an abundance of another TRAF, perhaps human TRAF2 or related molecules in cells that can intermediate between LMP1 and EBI6. This raises the possibility that the TRAFs may have a role as regulators of vesicle formation or transport which may be related or unrelated to their role in TNF receptor family signaling.

The interaction of LMP1 with TNF receptor signaling pathways may also be important in enabling EBV infected cells to evade host defense mechanisms in latent or lytic EBV infection. LMP1 is one of the few EBV genes expressed in both phases of the virus life cycle (Mann, et al., 1985; Rowe, et al., 1992). Several virus families appear to specifically target the TNF/lymphotoxin pathways presumably to avoid these immune cell mediators of cytotoxicity. Pox viruses produce soluble versions of the 80 kDa TNF receptor (Smith et al., 1991; Massung, et al., 1993). Proteins encoded by the adenovirus E3 region block the apoptotic function of TNF (Gooding, 1992) and HIV utilizes NF-kB activating signals induced by TNF signaling to enhance transcription (Poli, et al., 1990). The binding of EBV LMP1 to LAP1/EBI6 may effectively compete with normal LAP1 binding to the 60kDa TNF receptor, blocking the induction of cell death mediated by that receptor (Tartaglia, et al., 1993b) or

III. Screens For Inhibitors of TRAF-Mediated Functions

As detained elsewhere, our invention particularly enables screens for inhibitors TRAF-mediated signal pathways.

1. *In vitro* screens

In vitro screens may involve immobilizing one member of the interacting pair (or a relevant fragment thereof) on a substrate (e.g., the wells of a microtitre plate or beads used in a column). The immobilized member is then contacted with the other member of the interacting pair, which may be labeled. Binding is detected by detecting label associated with the substrate after washing unbound label away. In the absence of an inhibitor, this protein/protein interaction will yield bound label. Inhibitors of the protein-protein interaction will reduce the amount of bound label. Alternatively, competitive binding formats may be used, in which both binding partners are presented in the presence of candidate inhibitors, primarily competitive binding inhibitors.

Those skilled in the art will appreciate that there can be a large number of variations in the details of the above-described format. For example, polypeptide fragments can be attached to microtitre wells or beads by several well-known techniques. Labels including radioactive,

fluorescent, and enzymatic labels may be used. Alternatively, protein/protein interaction can be measured, electrically, e.g., using the BIOCORE® apparatus, (Pharmacia). Similarly, association between glutathione and glutathione binding polypeptides such as GST can be used to detect association of two proteins, as described in Figures 5A and 5B.

Those skilled in the art will understand that there are a large number of detailed formats for performing such *in vitro* screens. For example, optical systems, such as Amersham's fluorescent pair (inhibitor-coupled) readout, may be used to detect interactions that bring fluorescent pair members in association so as to generate a fluorescent signal. BIOCORE's electrical signal generating apparatus can be used to detect direct interaction.

Screening assays for inhibitors of TRAF interactions are based on procedures for detecting binding interactions, which then serve as controls for screens in which the candidate inhibitor is added. Procedures for detecting binding interactions may be carried out using recombinant receptor proteins produced by engineered cells.

Candidate ligands may be purified (or substantially purified) molecules or the ligand may be one component of a mixture of ligands (e.g., an extract or supernatant obtained from cells. The ligand may also be identified by testing progressively smaller subsets of the ligand pool (e.g., produced by standard purification techniques, e.g., HPLC or FPLC) until a single ligand is finally demonstrated to modulate the activity in question. Candidate ligands include peptide as well as non-peptide molecules.

Alternatively, a ligand (and an inhibitor) may be identified by its ability to bind using affinity chromatography. Recombinant binding partner is purified by standard techniques, from cells engineered to express it. The recombinant partner immobilized on a column (e.g., a Sepharose column or a streptavidin-agarose column by immunoaffinity methods) and a solution containing one or more candidate ligands is passed through the column. Again, candidate ligands include peptide as well as non-peptide molecules. A ligand specific for TRAF is immobilized on the column (because of its interaction with the TRAF). To isolate the ligand, the column is first washed to remove non-specifically bound molecules, and the ligand of interest is then released from the column and collected.

Ligands isolated from cells or biological fluids by the above methods (or any other appropriate method) may, if desired, be further purified (e.g., by high performance liquid chromatography). Once isolated in sufficiently-purified form, a novel peptide or non-peptide ligand may be partially sequenced (by standard amino acid sequencing techniques). From this partial amino acid sequence, a partial nucleic acid sequence is deduced which allows the preparation of primers for PCR cloning of the ligand gene. The TRAF, LMP1 or TNFR cytoplasmic segment which mediates interaction can be immobilized on a BIOCORE® plate and agents that inhibit interaction can be detected.

To immunologically detect a TRAF-binding molecule on the Western blot, a typical competitive antibody binding procedure can be employed, using an alkaline phosphatase-based detection protocol. Isolation of the TRAF genes also facilitates the identification of molecules which bind thereto and which may be useful as therapeutics, by providing ready sources of the full

molecules and any desired fragments thereof, using standard recombinant DNA expression techniques.

2. *In vivo* screens

In an alternative system, cultured cells may be used to detect association of two proteins, as exemplified by the yeast two hybrid system described in more detail below. The presence of β-galactosidase activity indicates association of a GAL4 activating domain and a GAL4 binding domain. These two domains can be functionally associated by fusing one to a TRAF domain and the other to a TRAF-associating domain, as described below.

Other *in vivo* systems include cultures of EBV-infected cells. For example, EBV-immortalized lymphoblastoid cells lines (LCL's) may be co-cultivated with primary B lymphocytes. Lytic infection is induced (e.g., with phorbol ester). Second generation LCL's are then recovered. Cell growth of the recovered cells and the presence of EBV DNA in those cells are measured as indicative of LMP1-mediated activities.

As noted elsewhere, candidate inhibitors include polypeptide fragments of LMP1, and LAP1 and EBI6. Inhibitor fragments may be used to design and produce non-peptide drugs that retain the inhibitory function. Candidate non-peptide drugs then may be screened as described above.

Brief Description of the Drawings

We will briefly describe the Drawings and then we will describe the specific examples related thereto.

Figure 1 depicts alignment of the amino acid sequences of human EBI6 and LAP1 and murine TRAF1 and TRAF2.

Figures 2A-2D are RNA blots described below.

Figure 3 shows intracellular association of LMP1 with LAP1 or EBI6 in transfected BJAB, non-EBV-infected, B lymphoma cells.

Figures 4A-4P are photos showing subcellular localization of LAP1 and EBI6 in the presence or absence of LMP1.

Figures 5A-5C show association of LAP1 and EBI6 with TNFR related proteins.

Figures 6A and 6B are schematic representations of TRAF mediated signal transduction.

Examples

I. A yeast two hybrid screen reveals proteins that interact with the LMP1 carboxy terminal cytoplasmic domain.

DNA encoding the 200 amino acid LMP1 carboxy terminal cytoplasmic domain was fused in frame to the GAL 4 DNA binding domain for use as bait in a yeast two hybrid screen for cDNAs that encode interactive proteins. The GAL 4 activating domain was fused to cDNAs made from RNA from EBV transformed B lymphocytes (Durfee, et al., 1993). Of $5X10^5$ transformants which were tested for growth in the absence of tryptophane, leucine and histidine and in the presence of 25mM 3-aminotriazole, 147 colonies showed at least moderate growth and were analyzed for β-galactosidase expression. Two clones were strongly positive for β-galactosidase, scoring higher than 8 units in a standard β-galactosidase assay, whereas the rest of the clones had nearly background levels of β-galactosidase activity (less than 0.04 units). The GAL 4 activating domain-cDNA gene fusions from these two clones did not interact with GAL4 DNA-binding domain fusions to p53, to pRB, to lamin, or to yeast SNF1 protein indicating specificity for the LMP1 cytoplasmic carboxy terminus.

From the sequence of the complete open reading frame, full length LAP1 has an amino terminal RING finger metal binding motif and a carboxy terminal domain that begins with an extended coiled coil motif (Figure 1). The carboxy terminal LAP1 domain (amino acids 302-568) has collinear 45% amino acid identity to the "TRAF" homology domain of the recently identified murine tumor necrosis factor (TNF) receptor associated proteins, TRAF1 and TRAF2 (Rothe, et al., 1994) (Figure 1). LAP1 is similar to TRAF2 in having an amino terminal RING finger motif but is only 27% identical to TRAF2 overall. The longest open reading frame identified in the alternatively spliced LAP1 mRNA encodes for a polypeptide that initiates at methionine codon 350 within the coiled coil motif of full length LAP1 and includes the rest of the TRAF domain. Since amino acids 345-568 of LAP1 interact strongly with the LMP1 carboxy terminal cytoplasmic domain (Table 1), the protein encoded by the spliced LAP1 mRNA could positively or negatively modulate interactions of LAP1 or LMP1.

II. The carboxy terminal 223 amino acids of LAP1 interact strongly with the membrane proximal 44 amino acids of the carboxy terminal cytoplasmic domain.

The full LMP1 cytoplasmic carboxy terminus (amino acids 187-386) interacts strongly with the LAP1 carboxy terminal 386 or 223 amino acids, although the interaction with the LAP1 223 amino acid carboxy terminal domain may be somewhat weaker (Table 1). The apparently essential membrane proximal 44 amino acids of LMP1 (amino acids 188 to 231) interact strongly with the LAP1 carboxy terminal 386 or 223 amino acids (Table 1). Thus, the LMP1 membrane proximal 44 amino acids and the LAP1 carboxy terminal 223 amino acids encompass major components of the LMP1-LAP1 interface. The apparently essential role

of the membrane proximal 44 amino acids of LMP1 in transformation genetically links the LMP1-LAP1 biochemical interaction to LMP1 mediated transformation.

III. Plasmid construction

The following genetic constructions were used to clone LAP1 and EBI6 using the yeast two hybrid system.

The GAL 4 DNA binding domain (G4DBD) fusions were constructed in vector pAS2 (Harper, et al., 1993). G4DBDLMP1(187-386) was constructed by polymerase chain reaction (PCR) mediated amplification of the LMP1 cDNA fragment encoding amino acids 187-386 using oligos L1-5PCR (5'-CGCGGATCCATGGACAACGACACAGTG-3') and L1-4PCR (5'-CGCGGATCCTTAGTCATAGTAGCTTAG-3') followed by cloning into the BamHI site of pAS2. G4DBDLMP1(187-231) was constructed by PCR-amplification of the LMP1 cDNA fragment encoding amino acids 187-231 using oligos L1-5PCR and LCA231 (5'-CGCGGATCCTTAGGCTCCACTCACGAGCAG-3') followed by cloning into the BamHI site of pAS2. G4DBDLAP1(12-568) was constructed by isolating the BssHII-BamHI fragment of LAP1 cDNA from pSG5LAP1, blunt-ending it using T4 DNA polymerase and subcloning it into the SmaI site of pAS1. G4TADEBI6(53-416) was constructed by subcloning the BglII fragment of EBI6 cDNA into the BamHI site of pACTII (a kind gift of S. Elledge). G4TADEBI6(53-416) encodes for an in frame fusion of EBI6 amino acids 53-416 to the acidic transactivating domain of GAL 4. G4TADLAP1(183-568) and G4TADLAP1(345-568) were isolated from the two-hybrid screening. cDNA inserts from clones were subcloned into the EcoRI site of plasmid pSG5 for sequencing analysis. pSG5 subclones of cDNA clones were spliced at the NruI site to generate full length LAP1 expressing construct pSG5LAP1. The EcoRI insert of λgt10 clone EBI6 was subcloned into plasmid pBluescript for sequencing analysis. pSG5FLAGLAP1 and

pSG5FLAGEBI6 were constructed in vector pSG5 by placing through PCR a FLAG-encoding DNA fragment right after the initiator AUG codon.

IV. Subtractive hybridization

Construction of the λgt10 cDNA library from the EBV-positive cell line BL41/B95-8 was previously described (Birkenbach, et al., 1993). Subtractive hybridization and homology screening of a λgt10 library was done as described before (Birkenbach, et al., 1993).

V. Yeast two-hybrid screening

Reagents necessary for culturing yeast were bought from BIO101. Yeast transformation was performed according to the method of Schiestl and Geitz (Schiestl and Gietz, 1989). The yeast strain Y190 (Durfee, et al., 1993) was transformed with plasmid construct G4DBDLMP1(187-386), and transformants were selected on SC-Trp plates. A single colony was picked and the expression of the LMP1 fusion protein was verified by Western blotting using the S12 anti-LMP1 monoclonal antibody. The G4DBDLMP1(187-386) transformant was subsequently transformed with a cDNA library constructed previously from an EBV-transformed lymphoblastoid cell line (Durfee, et al., 1993) and selection was done on SC media lacking tryptophan, leucine and histidine in the presence of 25 mM 3-aminotriazole (Sigma) as previously described (Durfee, et al., 1993). Colonies that showed moderate to intense growth were streaked on SC-Trp,Leu,His plates containing 50 mM 3-aminotriazole and tested for β-galactosidase expression by a filter lift assay (Breeden and Nasmyth, 1985). For quantitation of lacZ expression, yeast clones were grown in appropriate selective media to OD_{600} of 0.5-1.2 and assayed for β-galactosidase activity using o-nitrophenyl-β-D-galactoside (ONPG) and standard conditions as previously described (Breeden and Nasmyth, 1985). β-galactosidase

units were expressed as $(1000A_{415})/(\text{assay time in minutes})(\text{cell culture volume in milliliters})(\text{cell culture optical density at 600 nm})$. Library derived plasmids were recovered by transformation of competent bacteria with total yeast DNA preps followed by selection for ampicillin resistance as previously described (Ausubel, et al., 1987).

VI. Northern Blots

As shown in Figures 2A-2D, Northern blots containing polyA+ RNA (2 μg per lane) from eight human tissues were purchased from Clontech. RNA was prepared from EBV-positive (BL41/B95-8) or EBV-negative (BL41) Burkitt's lymphoma cell lines and a lymphoblastoid cell line (IB4) as previously described (Birkenbach, et al., 1993). cDNA probes were labeled by random hexanucleotide priming (Stratagene) using ^{32}P-dCTP. The RNA blots were hybridized to ^{32}P-labeled cDNA probes, under high stringency conditions as described (Mosialos, et al., 1994). Northern blot filters were exposed to autoradiography film or processed by phosphorimager analysis.

Specifically in Figures 2A and 2B, the RNA is poly(A+)RNA from human tissues. In Figure 2C, the RNA is poly(A+)RNA from cell lines, and, in Figure 2D, the RNA is total cell line RNA. The blots were hybridized to LAP1 (2A and 2C) or EBI6 (2B and 2D) probes, shown below the blot. The origin of RNA is shown above each lane with the following designations -- PA: pancreas, KI: kidney, SM: skeletal muscle, LI: liver, LU: lung, PL: placenta, BR: brain, HE: heart. Size markers are to the left of each blot and arrows indicate the position of specifically and consistently detected mRNAs. The LAP1 probe detected 2.8 and 1.8 Kb RNAs whereas EBI6 probe detected a 2.6 Kb RNA. The high molecular weight bands were not consistently detected in other northern blots

with these probes. LAP1 (2C) and EBI6 (2D) mRNAs were also detected in RNA from EBV infected BL41 (BL41/B95-8), EBV negative BL41 (BL41), and EBV transformed (IB4) cells. An actin probe (ACTIN) indicates the relative amounts of RNA in Figures 2C and 2D.

VII. Immunoprecipitations, Western Blotting and immunofluorescence

The following general technique illustrates to a method for determining intracellular protein-protein interaction. BJAB cells were electroporated at 220 V and 960 μF in 400 μl of RPMI-1640 medium containing 10% fetal calf serum. Approximately 20 hours post-transfection cells were lysed for 30 min on ice in 0.5% NP-40 lysis buffer containing 50 mM HEPES (pH 7.4), 250 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml pepstatin A and 2 μg/ml leupeptin. Cell debris were removed by centrifugation at 10,000 X g for 10 min at 4°C. The cell lysates were precleared with protein G-sepharose beads for 1 hour at 4°C. The primary antibody was then added for 1 hour at 4°C and immunoglobulin complexes were collected on protein G-sepharose beads for 1 hour at 4°C. The beads were then washed six times with 1ml of lysis buffer each time and protein complexes were recovered by boiling in SDS sample and analyzed by SDS-PAGE. Western blotting was done using standard techniques as previously described (Mosialos, et al., 1994).

Indirect immunofluorescence analysis on transfected cells was done approximately 18 to 20 hours post-transfection as previously described (Mosialos, et al., 1994).

VIII. Intracellular association of LMP1 with LAP1 or EBI6 in transfected BJAB, non-EBV-infected, B lymphoma cells.

BJAB cells (10 x 10^6 cells per transfection) were electroporated with plasmids expressing the proteins indicated by + at the bottom of the figure. Approximately 20 hours post transfection 4 x 10^6 cells from each transfection were lysed and subjected to immunoprecipitation with 10 μg of M2 anti-FLAG monoclonal antibody (Kodak). Results are shown in Figure 3. Equivalent cell lysates obtained before immunoprecipitation (lanes 1-4) and immunoprecipitated material (lanes 5-8) were analyzed by SDS polyacrylamide electrophoresis on a 7.5% gel, transferred onto nitrocellulose and subjected to western blot analysis using rabbit anti-LMP1 polyclonal antisera (Hennessy et al., 1984) and ^{125}I-labeled protein A followed by autoradiography. The position of LMP1 is shown by the arrow and molecular weight markers are shown on the right side of the panel. LMP1 was readily coimmunoprecipitated with FLAG1AP1 or FLAGEBI6 (lanes 6 and 7). No detectable LMP1 was seen in anti-FLAG immunoprecipitations from cells cotransfected with pSG5LMP1 and either vector control (pSG5) or a construct expressing a FLAG-tagged EBNA2 (FLAGE2, lanes 5 and 8).

IX. Production and purification of GST fusion proteins

The cytoplasmic domains of the p80 and p60 TNF receptors were amplified from the corresponding cDNAs by PCR and were cloned in-frame into the pGEX-4T-1 expression vector (Pharmacia) using the BamHI and XhoI restriction sites for the p60 TNF receptor and the EcoRI and XhoI sites for the p80 TNF receptor. Expression and purification of GST-fusion proteins were performed essentially as described previously (Smith and Johnson, 1988). Fusion protein concentrations of 3-5 mg per milliliter of glutathione-agarose beads (Pharmacia) were

routinely obtained. In vitro translations were done using the rabbit reticulocyte TNT coupled in vitro transcription translation system (Promega) according to manufacturer's protocol. In vitro translated proteins were diluted with binding buffer (PBS containing 0.1% NP-40, 0.5 mM DTT, 10% glycerol, 1 mM PMSF and 2 µg/ml aprotinin) and precleared with glutathione beads for 45 min at 4°C. GST or GST fusion proteins bound to glutathione beads were then incubated with in vitro translated proteins for 1 hour at 4°C. The beads were subsequently washed 5 times with 0.5 ml of binding buffer each time and bound proteins were recovered by boiling in SDS sample buffer and analyzed by SDS-PAGE.

X. Subcellular localization.

In Figures 4A-4P, the intracellular distribution of FLAG-tagged LAP1 and EBI6 was determined by indirect immunofluorescence using M2 anti-FLAG monoclonal antibody and rabbit anti LMP1 polyclonal antisera. BJAB cells were transfected with FLAGLAP1 (Figures 4A, 4B and 4E-4J) or FLAGEBI6 (Figures 4C, 4D and 4K-4P) expressing constructs in the presence of vector pSG5 (Figures 4A-4D) or pSG5LMP1 (Figures 4E-4P). M2 anti-FLAG reactivity was visualized with a FITC-conjugated goat anti-mouse secondary antibody (Figures 4A, 4C, 4E, 4H, 4K, 4N). LMP1 was detected with a Texas Red-conjugated goat anti rabbit secondary antibody (Figures 4F, 4I, 4L, 4O). Phase contrast pictures are shown in Figures 4B, 4D, 4G, 4J, 4M and 4P. M2 and anti-LMP1 antibodies did not show any reactivity in untransfected cells. No cross-reactivity was observed between M2 and the goat anti-rabbit secondary antibody or between the rabbit anti-LMP1 and goat anti-mouse secondary antibody (data not shown).

XI. Association of LAP1 and EBI6 with TNFR related proteins.

In Figures 5A-5C, we demonstrate association of LAP1 and EBI6 with the cytoplasmic domains of several

TNFRs. For example, the cytoplasmic domains of the p60 and/or p80 TNFR were constructed as fusion proteins with GST and bound to glutathione beads. These cytoplasmic domains thus bound were incubated with ^{35}S-methionine labeled LMP-1, LAP1 or EBI6 translated *in vitro* (5μl of reaction mix) and the fraction bound to glutathione beads was analyzed on a 8.5% SDS polyacrylamide gel and processed by a phosphorimager. Coomasie blue staining of the gel demonstrated the presence of approximately equivalent amounts of GST or GST-fusion proteins. In 5B, glutathione beads containing cytoplasmic domains of p60 (lane 3), p80 (lane 4), Fas (lane 5), CD40 (lane 6), LTβR (lane 7) expressed as GST fusion proteins or GST (lane 2) were incubated with ^{35}S-methionine labeled LAP1 (2μl of *in vitro* translation reaction mix were used per reaction) as in Figure 5A, and analyzed by SDS-PAGE and autoradiography (2 hr exposure). Two μl of *in vitro* translated LAP1 were analyzed in lane 1. Figure 5C shows co-immunoprecipitation of LAP1 and EBI6 with p80 TNFR in cotransfected cells. BJAB cells were cotransfected with plasmids expressing the FLAG tagged proteins indicated by a + at the bottom of the figure of left untransfected (lane 7). Approximately 20 hours post-transfection the cells were lysed and lysates from 10X10^6 cells were subjected to immunoprecipitation with M2 anti-FLAG monoclonal antibody. Equivalent cell lysates obtained before immunoprecipitation (lanes 4-7) and immunoprecipitated complexes (lanes 1-3) were analyzed by Western blotting using an anti-p80 TNFR antibody (Van Arsdale and Ware 1994). The position of mature p80 TNFR and the immunoglobulin heavy chain (Ig) are shown by arrows. The star shows the position of a precursor form of the p80 TNFR. The p80 receptor was readily coimmunoprecipitated with FLAGLAP1 or FLAGEBI6 (lanes 1 and 2). No detectable p80 receptor was

immunoprecipitated with anti-FLAG antibody from cells cotransfected with plasmids expressing p80 TNFR and FLAGEBNA2 (FLAGE2, lane 3).

Other Embodiments

As detailed above, the invention includes substantially pure proteins and polypeptides. We use the term polypeptide to refer to any peptide bond-containing molecule without limitation on size, including proteins and shorter polypeptides. A protein or polypeptide is substantially pure when it is separated from those contaminants which accompany it in its natural state. As we use the term, a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be "substantially free" from its naturally associated components. Accordingly, substantially pure polypeptides include those derived from eukaryotic organisms but synthesized in *E. coli* or other prokaryotes.

The invention also includes a substantially pure nucleic acid which hybridizes at high stringency to a nucleic acid encoding LAP1. By "hybridizes" is meant binds to or associates with a nucleic acid of specified sequence. By the term "high stringency" is meant DNA hybridization and wash conditions characterized by relatively high temperature and low salt concentration, e.g., conditions described in Sambrook et al., 1989, *Molecular Cloning: a Laboratory Manual*, second edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., e.g., 0.2 × SSC, 0.1% SDS at 60 °C wash conditions.

By "substantially pure DNA" is meant DNA that is free of the genes which flank the gene in the naturally-occurring genome of the organism from which the DNA of the invention is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or

virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By "promoter" is meant minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific or inducible by external signals or agents; such elements may be located in the 5′ or 3′ regions of the native gene.

By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

Degenerate variants of the nucleic acid encoding LAP1 or EBI6 and other polypeptides described above are also within the invention. Degenerate variants are nucleic acids which encode a polypeptide with the amino acid sequence of LAP1 or EBI6, but differ in nucleotide sequence from the cDNA sequences disclosed herein.

As used herein, the term "substantially pure" describes a protein or polypeptide, which has been separated from components which naturally accompany it. Typically, a protein or polypeptide is substantially pure when at least 10%, more preferably at least 20%, more preferably at least 50%, more preferably at least 60%, more preferably at least 75%, more preferably at least 90%, and most preferably at least 99%, of the total material (by volume, by wet or dry weight, or by mole per cent or mole fraction) in a sample is the protein or

polypeptide of interest. Purity can be measured by any appropriate method, e.g., in the case of polypeptides by column chromatography, polyacrylamide gel electrophoresis, or high pressure liquid chromatographic (HPLC) analysis.

The invention also includes a biologically active fragment of LAP1 or EBI6. By the term "biologically active" is meant having the ability to control TRAF-mediated events such as EBV-induced phenotype traits. As used herein, the term "fragment or segment", as applied to a polypeptide, will ordinarily be at least about 5 contiguous amino acids, typically at least about 10 contiguous amino acids, more typically at least about 20 contiguous amino acids, usually at least about 30 contiguous amino acids, preferably at least about 40 contiguous amino acids, more preferably at least about 50 contiguous amino acids, and most preferably at least about 60 to 80 or more contiguous amino acids in length. Such peptides can be generated by methods known to those skilled in the art, including proteolytic cleavage of the protein, *de novo* synthesis of the fragment, or genetic engineering.

In another aspect, the invention features an antibody which specifically binds to LAP1 or EBI6.

The invention also includes homologous human LAP proteins and DNA encoding them. For example, it includes proteins more than 50% homologous to the LAP1 sequences (SEQ ID NO: 1) or active fragments thereof. "Homology", as used herein, refers to the subunit sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, e.g., two DNA molecules, or two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that

position. The homology between two sequences is a direct function of the number of matching or homologous positions, e.g., if half (e.g., 5 positions in a polymer 10 subunits in length), of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, e.g., 9 of 10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3'ATTGCC'5 and 3'TATGGC'5 share 50% homology.

Recombinant LAP1 or EBI6 or any fragment thereof (e.g., a biologically active domain) can be expressed using known methods. DNA sequences encoding LAP1 or EBI6 can be cloned into commercially available expression vectors and expressed in *E. coli*.

For example, the maltose binding protein fusion and purification system (New England Biolabs) can be used to overexpress the fusion protein. The LAP1 or EBI6 gene or cDNA can be inserted downstream and in frame of the gene encoding maltose binding protein (malE). In the absence of convenient restriction sites, PCR can be used in order to appropriately modify the cDNA sequence. This well known method can facilitate construction of the recombinant plasmid. Immediately upstream of the insertion site of the pMalE plasmid is region encoding a factor Xa cleavage site. The presence of this specific proteolytic-sensitive site allows liberation of the cloned protein from the maltose binding protein without additional amino acids attached at the N-terminus, an advantage over other methods for expressing and purifying recombinant proteins in bacteria. Using this expression system, the recombinant protein can be targeted to either the cytoplasm or periplasmic space, depending upon the presence or absence of the malE signal sequence. Purification of the fusion protein can be achieved by passing the crude cell lysate over an amylose resin

column, to which the malE fusion protein specifically binds. The eluted pure hybrid protein can then be cleaved by factor Xa and the protein of interest purified from maltose binding protein and factor Xa by standard column chromatography.

Other expression systems, e.g., the glutathione-S-transferase gene fusion system (Pharmacia), may also be used to express all or part of the LAP1 or EBI6 proteins. In this system, TRAF DNA sequences may be cloned into the appropriate vector, and fusion proteins expressed in *E. coli*. Purification of the resulting recombinant proteins is accomplished by standard column chromatography using glutathione Sepharose 4B beads.

Alternatively, LAP1 or EBI6 can be expressed using a eucaryotic expression system. Expression vectors and eucaryotic cells suitable for expressing recombinant proteins (e.g., mammalian cells, insect cells, yeast cells) are also well known in the art.

Antibody Production and Western Blotting

In order to identify the LAP1 or EBI6 polypeptide in cellular extracts and study its potential association with other molecules, antibodies which specifically bind to those proteins are useful. Synthetic peptides designed from the predicted LAP1 or EBI6 sequence and/or the purified polypeptide produced by bacterial or eucaryotic cells can be used as antigens to immunize animals for the production of polyclonal antisera using standard protocols.

Antibodies directed against specific antigens may be detected by any of several methods known to those skilled in the art, e.g., by using an Ouchterlony double diffusion assay or an enzyme-linked immunoabsorbent assay (ELISA). ELISA involves coating a substrate, e.g., well in a plastic dish, with a purified antigen. Serum to be

tested is then added to the well. If present, antigen specific antibodies attach to the antigen coating the well. Non-binding material is washed away and a marker enzyme e.g., horse radish peroxidase or alkaline phosphatase, coupled to a second antibody directed against the antigen-specific primary antibody is added in excess and the nonadherent material is washed away. Finally the enzyme substrate is added to the well and the enzyme catalyzed conversion is monitored as indicative of presence of the antigen.

To produce monoclonal antibodies, antibody-producing cells from the challenged animal can be immortalized (e.g., by fusion with an immortalizing fusion partner) to produce monoclonal antibodies. Monoclonal antibody-producing hybridomas can then be screened for antibody binding to the polypeptide as described above.

The invention can employ not only intact monoclonal or polyclonal antibodies, but also an immunologically-active antibody fragment, for example, a Fab or $(Fab)_2$ fragment; an antibody heavy chain, an antibody light chain; a genetically engineered single-chain Fv molecule (Ladner et al., U.S. Patent No. 4,946,778); or a chimeric antibody, for example, an antibody which contains the binding specificity of a murine antibody, but in which the remaining portions are of human origin.

The LAP1- or EBI6-specific antibodies can be employed in Western analyses in order to identify recombinant clones expressing the LAP1 or EBI6 gene product.

Peptide therapy

The purified polypeptides can be administered in a pharmaceutically acceptable carrier, e.g., physiological saline.

The invention includes analogs in which one or more peptide bonds have been replaced with an alternative type of covalent bond (a "peptide mimetic") which is not susceptible to cleavage by peptidases. Where proteolytic degradation of the peptides following injection into the subject is a problem, replacement of a particularly sensitive peptide bond with a noncleavable peptide mimetic will make the resulting peptide more stable and thus more useful as a therapeutic. Such mimetics, and methods of incorporating them into polypeptides, are well known in the art. Similarly, the replacement of an L-amino acid residue is a standard way of rendering the polypeptide less sensitive to proteolysis. Also useful are amino-terminal blocking groups such as t-butyloxycarbonyl, acetyl, theyl, succinyl, methoxysuccinyl, suberyl, adipyl, azelayl, dansyl, benzyloxycarbonyl, fluorenylmethoxycarbonyl, methoxyazelayl, methoxyadipyl, methoxysuberyl, and 2,4,-dinitrophenyl. Blocking the charged amino- and carboxy-termini of the peptides would have the additional benefit of enhancing passage of the peptide through the hydrophobic cellular membrane and into the cell.

The polypeptides can be administered intraperitoneally, intramuscularly, subcutaneously, or intravenously.

Standard methods for intracellular delivery of peptides can be used, e.g. with liposomes. Such methods are well known to those of ordinary skill in the art. It is expected that an intravenous dosage of approximately 1 to 100 μmoles of the peptide of the invention would be administered per kg of body weight per day.

Gene therapy

In some cases, patients may be treated by administering the nucleic acid of the invention, such

that the expression of recombinant polypeptide takes place in the cells, e.g., tumor cells, of the patient, such as tumor cells. The nucleic acid of the invention may be introduced into target cells of a patient by standard vectors and/or gene delivery systems. Suitable gene delivery systems include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, and adenoviruses, among others.

For treatment of patients, a therapeutically effective amount of a nucleic acid may be administered in a pharmaceutically acceptable carrier. Dosages for the nucleic acid molecules of the invention will vary, but a preferred dosage for intravenous administration is approximately from 10^6 to 10^{22} copies of the nucleic acid molecule.

For treatment, a therapeutically effective amount of a nucleic acid administered in a pharmaceutically acceptable carrier. A pharmaceutically acceptable carrier is a vehicle that is suitable, i.e., biologically compatible, for administration to an animal, e.g. physiological saline. A therapeutically effective amount is an amount of the nucleic acid of the invention which is capable of producing a medically desirable result in a treated animal.

As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosages for the nucleic acid molecules of the invention will vary, but a preferred

dosage for intravenous administration is approximately from 10^6 to 10^{22} copies of the nucleic acid molecule.

Once improvement of the patient's condition has occurred, a maintenance dose may be administered if necessary. Subsequently, the dosage or the frequency of administration, or both, may be reduced, as a function of the symptoms, to a level at which the improved condition is retained. When the symptoms have been alleviated to the desired level, treatment should cease. Patients may, however, require intermittent treatment on a long-term basis upon any recurrence of disease symptoms.

Also included in the invention are analogues of the native protein or polypeptides. Analogs can differ from the native peptides by amino acid sequence, or by modifications which do not affect the sequence, or by both.

Preferred analogs include peptides whose sequences differ from the wild-type sequence (i.e., the sequence of the homologous portion of the naturally occurring peptide) only by conservative amino acid substitutions, preferably by only one, two, or three, substitutions, for example, substitution of one amino acid for another with similar characteristics (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions which do not abolish the polypeptide's biological activity. Table 2 lists a number of conservative amino acid substitutions. Also included are chemically synthesized peptides with modified peptide bonds or modified side chains to obtain the desired pharmaceutical properties.

Modifications (which do not normally alter primary sequence) include *in vivo* or *in vitro* chemical derivitization of polypeptides, e.g., acetylation or carboxylation. Also included are modifications of

glycosylation, e.g., those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps, e.g., by exposing the polypeptide to enzymes which affect glycosylation e.g., mammalian glycosylating or deglycosylating enzymes. Also included are sequences which have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine

For treatment of localized tumors, a bio-polymer delivery system designed for the slow release of the polypeptide of the invention may be implanted in close proximity to the tumor mass. Such bio-polymer delivery systems are well known in the art (see, e.g., Folkman et al., U.S. Patent 4,164,560, herein incorporated by reference).

Table 1. 8.3 β-galactosidase assay of protein-protein interactions in the yeast two-hybrid system

Transformant	β-galactosidase units
G4DBDLMP1(187-386)+G4TADLAP1(183-568)	56
G4DBDLMP1(187-386)+G4TADLAP1(345-568)	8
G4DBDLMP1(187-231)+G4TADLAP1(183-568)	5
G4DBDLMP1(187-231)+G4TADLAP1(345-568)	5
G4DBDLMP1(187-386)+G4TADEBI6(53-416)	0.07
G4DBDLAP1(12-568)+ G4TADEBI6(53-416)	0.07
G4DBDLMP1(187-386)	0.04
G4DBDLMP1(187-231)	0.1
G4TADLAP1(183-568)	0.04
G4TADLAP1(345-568)	0.1
G4TADEBI6(53-416)	0.04
G4DBDLAP1(12-568)	0.05
G4DBDNSF1+G4TADSNF4	0.8

The yeast strain Y190 was transformed with the indicated plasmids and transformants were selected on appropriate selective defined media. Isolated colonies were grown to mid to late log density and assayed for β-galactosidase activity as described in experimental procedures. Four individual transformants were assayed in each case and the average values of β-galactosidase units are shown. The interaction between G4DBDLAP1 and G4TADSNF4 was used as a control (Harper et al. 1993) and scored 0.8 β-galactosidase units or higher in different assays.

TABLE 2

CONSERVATIVE AMINO ACID REPLACEMENTS

For Amino Acid	Code	Replace With
Alanine	A	D-Ala, Gly, Aib, β-Ala, Acp, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, Aib, β-Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, AdaA, AdaG, cis-3,4, or 5-phenylproline, Bpa, D-Bpa
Proline	P	D-Pro, L-I-thioazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid (Kauer, U.S. Patent (4,511,390)
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met, AdaA, AdaG

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toxicity but highly susceptible to infection by *Listeria monocytogenes*. Nature *364*, 798-802.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Brigham & Women's Hospital

(ii) TITLE OF INVENTION: CONTROLLING TRAF-MEDIATED SIGNALS

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Fish & Richardson
(B) STREET: 225 Franklin Street Suite 3100
(C) CITY: Boston
(D) STATE: MA
(E) COUNTRY: U.S.A.
(F) ZIP: 02110-2804

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30B

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US95/----
(B) FILING DATE:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/367,540
(B) FILING DATE: 30-DEC-1994

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Freeman, John W.
(B) REGISTRATION NUMBER: 29,066
(C) REFERENCE/DOCKET NUMBER: 05311/014WO1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617)542-5070
(B) TELEFAX: (617)542-8906
(C) TELEX: 100254

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2359 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 151..1854

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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                                        Met Glu Ser Ser Lys Lys Met Asp
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     10                  15                  20

CGC AGT GCT GGG ACG CCA GTT TTT GTC CCT GAA CAA GGA GGT TAC AAG   270
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 25                  30                  35                  40

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His Leu Val Leu Cys Ser Pro Lys Gln Thr Glu Cys Gly His Arg Phe
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Cys Glu Ser Cys Met Ala Ala Leu Leu Ser Ser Ser Ser Pro Lys Cys
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Thr Ala Cys Gln Glu Ser Ile Val Lys Asp Lys Val Phe Lys Asp Asn
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Cys Cys Lys Arg Glu Ile Leu Ala Leu Gln Ile Tyr Cys Arg Asn Glu
105                 110                 115                 120

AGC AGA GGT TGT GCA GAG CAG TTA ATG CTG GGA CAT CTG CTG GTG CAT   558
Ser Arg Gly Cys Ala Glu Gln Leu Met Leu Gly His Leu Leu Val His
                125                 130                 135

TTA AAA AAT GAT TGC CAT TTT GAA GAA CTT CCA TGT GTG CGT CCT GAC   606
Leu Lys Asn Asp Cys His Phe Glu Glu Leu Pro Cys Val Arg Pro Asp
            140                 145                 150

TGC AAA GAA AAG GTC TTG AGG AAA GAC CTG CGA GAC CAC GTG GAG AAG   654
Cys Lys Glu Lys Val Leu Arg Lys Asp Leu Arg Asp His Val Glu Lys
        155                 160                 165

GCG TGT AAA TAC CGG GAA GCC ACA TGC AGC CAC TGC AAG AGT CAG GTT   702
Ala Cys Lys Tyr Arg Glu Ala Thr Cys Ser His Cys Lys Ser Gln Val
    170                 175                 180

CCG ATG ATC GCG CTG CAG AAA CAC GAA GAC ACC GAC TGT CCC TGC GTG   750
Pro Met Ile Ala Leu Gln Lys His Glu Asp Thr Asp Cys Pro Cys Val
185                 190                 195                 200

GTG GTG TCC TGC CCT CAC AAG TGC AGC GTC CAG ACT CTC CTG AGG AGC   798
Val Val Ser Cys Pro His Lys Cys Ser Val Gln Thr Leu Leu Arg Ser
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GAG TTG AGT GCA CAC TTG TCA GAG TGT GTC AAT GCC CCC AGC ACC TGT   846
Glu Leu Ser Ala His Leu Ser Glu Cys Val Asn Ala Pro Ser Thr Cys
            220                 225                 230

AGT TTT AAG CGC TAT GGC TGC GTT TTT CAG GGG ACA AAC CAG CAG ATC   894
Ser Phe Lys Arg Tyr Gly Cys Val Phe Gln Gly Thr Asn Gln Gln Ile
        235                 240                 245

AAG GCC CAC GAG GCC AGC TCC GCC GTG CAG CAC GTC AAC CTG CTG AAG   942
Lys Ala His Glu Ala Ser Ser Ala Val Gln His Val Asn Leu Leu Lys
    250                 255                 260

GAG TGG AGC AAC TCG CTC GAA AAG AAG GTT TCC TTG TTG CAG AAT GAA   990
Glu Trp Ser Asn Ser Leu Glu Lys Lys Val Ser Leu Leu Gln Asn Glu
265                 270                 275                 280

AGT GTA GAA AAA AAC AAG AGC ATA CAA AGT TTG CAC AAT CAG ATA TGT  1038
Ser Val Glu Lys Asn Lys Ser Ile Gln Ser Leu His Asn Gln Ile Cys
                285                 290                 295

AGC TTT GAA ATT GAA ATT GAG AGA CAA AAG GAA ATG CTT CGA AAT AAT  1086
Ser Phe Glu Ile Glu Ile Glu Arg Gln Lys Glu Met Leu Arg Asn Asn
            300                 305                 310

GAA TCC AAA ATC CTT CAT TTA CAG CGA GTG ATA GAC AGC CAA GCA GAG  1134
Glu Ser Lys Ile Leu His Leu Gln Arg Val Ile Asp Ser Gln Ala Glu
        315                 320                 325

AAA CTG AAG GAG CTT GAC AAG GAG ATC CGG CCC TTC CGG CAG AAC TGG  1182
Lys Leu Lys Glu Leu Asp Lys Glu Ile Arg Pro Phe Arg Gln Asn Trp
    330                 335                 340

GAG GAA GCA GAC AGC ATG AAG AGC AGC GTG GAG TCC CTC CAG AAC CGC  1230
Glu Glu Ala Asp Ser Met Lys Ser Ser Val Glu Ser Leu Gln Asn Arg
345                 350                 355                 360

GTG ACC GAG CTG GAG AGC GTG GAC AAG AGC GCG GGG CAA GTG GCT CGG  1278
Val Thr Glu Leu Glu Ser Val Asp Lys Ser Ala Gly Gln Val Ala Arg
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AAC ACA GGC CTG CTG GAG TCC CAG CTG AGC CGG CAT GAC CAG ATG CTG  1326
Asn Thr Gly Leu Leu Glu Ser Gln Leu Ser Arg His Asp Gln Met Leu
            380                 385                 390

AGT GTG CAC GAC ATC CGC CTA GCC GAC ATG GAC CTG CGC TTC CAG GTC  1374
Ser Val His Asp Ile Arg Leu Ala Asp Met Asp Leu Arg Phe Gln Val
        395                 400                 405

CTG GAG ACC GCC AGC TAC AAT GGA GTG CTC ATC TGG AAG ATT CGC GAC  1422
Leu Glu Thr Ala Ser Tyr Asn Gly Val Leu Ile Trp Lys Ile Arg Asp
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425                 430                 435                 440

TAC AGC CAG CCT TTC TAC ACT GGT TAC TTT GGC TAT AAG ATG TGT GCC    1518
Tyr Ser Gln Pro Phe Tyr Thr Gly Tyr Phe Gly Tyr Lys Met Cys Ala
                445                 450                 455

AGG GTC TAC CTG AAC GGG GAC GGG ATG GGG AAG GGG ACG CAC TTG TCG    1566
Arg Val Tyr Leu Asn Gly Asp Gly Met Gly Lys Gly Thr His Leu Ser
            460                 465                 470

CTG TTT TTT GTC ATC ATG CGT GGA GAA TAT GAT GCC CTG CTT CCT TGG    1614
Leu Phe Phe Val Ile Met Arg Gly Glu Tyr Asp Ala Leu Leu Pro Trp
        475                 480                 485

CCG TTT AAG CAG AAA GTG ACA CTC ATG CTG ATG GAT CAG GGG TCC TCT    1662
Pro Phe Lys Gln Lys Val Thr Leu Met Leu Met Asp Gln Gly Ser Ser
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CGA CGT CAT TTG GGA GAT GCA TTC AAG CCC GAC CCC AAC AGC AGC AGC    1710
Arg Arg His Leu Gly Asp Ala Phe Lys Pro Asp Pro Asn Ser Ser Ser
505                 510                 515                 520

TTC AAG AAG CCC ACT GGA GAG ATG AAT ATC GCC TCT GGC TGC CCA GTC    1758
Phe Lys Lys Pro Thr Gly Glu Met Asn Ile Ala Ser Gly Cys Pro Val
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TTT GTG GCC CAA ACT GTT CTA GAA AAT GGG ACA TAT ATT AAA GAT GAT    1806
Phe Val Ala Gln Thr Val Leu Glu Asn Gly Thr Tyr Ile Lys Asp Asp
            540                 545                 550

ACA ATT TTT ATT AAA GTC ATA GTG GAT ACT TCG GAT CTG CCC GAT CCC    1854
Thr Ile Phe Ile Lys Val Ile Val Asp Thr Ser Asp Leu Pro Asp Pro
        555                 560                 565

TGATAAGTAG CTGGGGAGGT GGATTTAGCA GAAGGCAACT CCTCTGGGGG ATTTGAACCG  1914

GTCTGTCTTC ACTGAGGTCC TCGCGCTCAG AAAAGGACCT TGTGAGACGG AGGAAGCGGC  1974

AGAAGGCGGA CGCGTGCCGG CGGGAGGAGC CACGCGTGAG CACACCTGAC ACGTTTTATA  2034

ATAGACTAGC CACACTTCAC TCTGAAGAAT TATTTATCCT TCAACAAGAT AAATATTGCT  2094

GTCAGAGAAG GTTTTCATTT TCATTTTTAA AGATCTAGTT AATTAAGGTG GAAAACATAT  2154

ATGCTAAACA AAAGAAACAT GATTTTTCTT CCTTAAACTT GAACACCAAA AAAACACACA  2214

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2380 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 76..1323

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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                 Met Ala Ser Ser Ser Gly Ser Ser Pro Arg Pro Ala
                     570                 575                 580

CCT GAT GAG AAT GAG TTT CCC TTT GGG TGC CCT CCC ACC GTC TGC CAG      159
Pro Asp Glu Asn Glu Phe Pro Phe Gly Cys Pro Pro Thr Val Cys Gln
                 585                 590                 595

GAC CCA AAG GAG CCC AGG GCT CTC TGC TGT GCA GGC TGT CTC TCT GAG      207
Asp Pro Lys Glu Pro Arg Ala Leu Cys Cys Ala Gly Cys Leu Ser Glu
             600                 605                 610

AAC CCG AGG AAT GGC GAG GAT CAG ATC TGC CCC AAA TGC AGA GGG GAA      255
Asn Pro Arg Asn Gly Glu Asp Gln Ile Cys Pro Lys Cys Arg Gly Glu
         615                 620                 625

GAC CTC CAG TCT ATA AGC CCA GGA AGC CGT CTT CGA ACT CAG GAG AAG      303
Asp Leu Gln Ser Ile Ser Pro Gly Ser Arg Leu Arg Thr Gln Glu Lys
     630                 635                 640

GCT CAC CCC GAG GTG GCT GAG GCT GGA ATT GGG TGC CCC TTT GCA GGT      351
Ala His Pro Glu Val Ala Glu Ala Gly Ile Gly Cys Pro Phe Ala Gly
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GTC GGC TGC TCC TTC AAG GGA AGC CCA CAG TCT GTG CAA GAG CAT GAG      399
Val Gly Cys Ser Phe Lys Gly Ser Pro Gln Ser Val Gln Glu His Glu
                 665                 670                 675

GTC ACC TCC CAG ACC TCC CAC CTA AAC CTG CTG TTG GGG TTC ATG AAA      447
Val Thr Ser Gln Thr Ser His Leu Asn Leu Leu Leu Gly Phe Met Lys
             680                 685                 690

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Gln Trp Lys Ala Arg Leu Gly Cys Gly Leu Glu Ser Gly Pro Met Ala
         695                 700                 705

CTG GAG CAG AAC CTG TCA GAC CTG CAG CTG CAG GCA GCC GTG GAA GTG      543
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     710                 715                 720

GCG GGG GAC CTG GAG GTC GAT TGC TAC CGG GCA CCC TGC TCC GAG AGC      591
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Gln Glu Glu Leu Ala Leu Gln His Phe Met Lys Glu Lys Leu Leu Ala
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GAG CTG GAG GGG AAG CTG CGT GTG TTT GAG AAC ATT GTT GCT GTC CTC      687
Glu Leu Glu Gly Lys Leu Arg Val Phe Glu Asn Ile Val Ala Val Leu
            760                 765                 770

AAC AAG GAG GTG GAG GCC TCC CAC CTG GCC CTG GCC ACC TCT ATC CAC      735
Asn Lys Glu Val Glu Ala Ser His Leu Ala Leu Ala Thr Ser Ile His
        775                 780                 785

CAG AGC CAG CTG GAC CGT GAG CGC ATC CTG AGC TTG GAG CAG AGG GTG      783
Gln Ser Gln Leu Asp Arg Glu Arg Ile Leu Ser Leu Glu Gln Arg Val
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GTG GAG CTT CAG CAG ACC CTG GCC CAG AAA GAC CAG GCC CTG GGC AAG      831
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805                 810                 815                 820

CTG GAG CAG AGC TTG CGC CTC ATG GAG GAG GCC TCC TTC GAT GGC ACT      879
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TGT GGC AGG ACC GTC AGC CTC TTC TCC CCA GCC TTC TAC ACT GCC AAG      975
Cys Gly Arg Thr Val Ser Leu Phe Ser Pro Ala Phe Tyr Thr Ala Lys
        855                 860                 865

TAT GGC TAC AAG TTG TGC CTG CGG CTG TAC CTG AAT GGA GAT GGC ACT     1023
Tyr Gly Tyr Lys Leu Cys Leu Arg Leu Tyr Leu Asn Gly Asp Gly Thr
    870                 875                 880

GGA AAG AGA ACC CAT CTG TCG CTC TTC ATC GTG ATC ATG AGA GGG GAG     1071
Gly Lys Arg Thr His Leu Ser Leu Phe Ile Val Ile Met Arg Gly Glu
885                 890                 895                 900

TAT GAT GCG CTG CTG CCG TGG CCC TTC CGG AAC AAG GTC ACC TTC ATG     1119
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                905                 910                 915

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GAC CTA AGC TCA GCG TCC TTC CAG AGG CCC CAG AGT GAA ACC AAC GTG     1215
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950 955 960

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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            20                  25                  30

Asn Glu Gly Arg His His Leu Leu Val Ser Gly Ala
        35                  40
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What is claimed is:

1. A method of treating Epstein-Barr virus (EBV)-associated infection, cell growth or tumorigenesis by administering to EBV-infected cells a compound that inhibits TRAF/TNFR-mediated cell growth/death signal transduction.

2. A medicament for treating Epstein-Barr virus (EBV)-associated infection, cell growth or tumorigenesis, said medicament comprising a compound that inhibits TRAF/TNFR-mediated cell growth/death signal transduction.

3. A method of making a medicament for treating Epstein-Barr virus (EBV)-associated infection, cell growth or tumorigenesis, said medicament comprising a compound that inhibits TRAF/TNFR-mediated cell growth/death signal transduction.

4. A method of treating Epstein-Barr virus (EBV)-associated infection, cell growth or tumorigenesis by administering to EBV-infected cells a compound that inhibits interaction between EBV LMP1 and a Tumor necrosis factor Receptor Associated Factor (TRAF) protein.

5. A medicament for treating Epstein-Barr virus (EBV)-associated infection, cell growth or tumorigenesis, said medicament comprising a compound that inhibits interaction between EBV LMP1 and a Tumor necrosis factor Receptor Associated Factor (TRAF) protein.

6. A method of making a medicament for treating Epstein-Barr virus (EBV)-associated infection, cell growth or tumorigenesis, said medicament comprising a compound that inhibits interaction between EBV LMP1 and a

Tumor necrosis factor Receptor Associated Factor (TRAF) protein.

7. The method of claim 4 or claim 6, or the medicament of claim 5, in which the compound is a polypeptide that interacts with EBV LMP1 protein.

8. The method or medicament of claim 7 in which the polypeptide includes an LMP1-interacting TRAF domain.

9. The method or medicament of claim 7 in which the polypeptide interacts with a region of the LMP1 carboxy terminus between amino acids 188 and 386.

10. The method or medicament of claim 7 in which the polypeptide interacts with the LMP1 sequence that extends from Gly188 to Ala231: G Q R H S D E H H H D D S L P H P Q Q A T D D S G H E S D S N S N E G R H H L L V S G A (SEQ ID NO:3).

11. The method or medicament of claim 8 in which the polypeptide includes a LMP1-interacting domain of a human TRAF protein.

12. The method or medicament of claim 11 in which the polypeptide includes a LMP1-interacting domain of LAP1.

13. The method or medicament of claim 12 in which the polypeptide includes a LMP1-binding domain within the LAP1 sequence between amino acids 345 and 568 of SEQ ID NO:1.

14. The method of claim 4 or claim 6, or the medicament of claim 5, in which the compound is a polypeptide that interacts with a TRAF protein.

15. The method or medicament of claim 14 in which the polypeptide includes a LMP1 sequence that interacts with a TRAF protein.

16. The method or medicament of claim 15 in which the polypeptide includes a LMP1 sequence that interacts with LAP1.

17. The method or medicament of claim 16 in which the polypeptide includes the sequence: G Q R H S D E H H H D D S L P H P Q Q A T D D S G H E S D S N S N E G R H H L L V S G A (SEQ ID NO:3).

18. The method or medicament of claim 14 in which the polypeptide interacts with a LMP1-binding domain within the LAP1 sequence between amino acids 345 and 568 of SEQ ID NO:1.

19. The method or medicament of claim 14 in which the polypeptide includes a human LAP oligomer-forming domain.

20. The method or medicament of claim 19 in which the polypeptide includes the following LAP1 sequence (amino acids 309-341 of SEQ ID NO:1):

L R N N E S K I L H L Q R V I D S
Q A E K L K E L D K E I R P F R

21. The method or medicament of claim 14 in which the polypeptide includes a TRAF oligomer-forming domain.

22. The method of claim 1, claim 3, claim 4, claim 6, or the medicament of claim 2 or claim 5, in which the compound is administered to a patient characterized by one or more of the following conditions: a) EBV infection; b) HIV infection; c) drug induced immunosuppression; d) Hodgkin's disease, e) Burkitt's lymphoma, f) a lymphoma characteristic of an immunocompromised patient, and g) nasopharyngeal carcinoma.

II. Controlling TRAF-Mediated TNF/TNFR Signaling

23. A method of controlling TRAF-Mediated TNF/TNFR signal transduction by administering to a TRAF-encoding cell a compound that inhibits TRAF signal transduction.

24. A medicament for controlling TRAF-Mediated TNF/TNFR signal transduction, said medicament comprising a TRAF-encoding cell a compound that inhibits TRAF signal transduction.

25. A method of making a medicament for controlling TRAF-Mediated TNF/TNFR signal transduction, said medicament comprising a TRAF-encoding cell a compound that inhibits TRAF signal transduction.

26. The method of claim 23 of claim 25, or the medicament of claim 24 in which the compound is a TRAF-interacting polypeptide.

27. The method or medicament of claim 26 in which the polypeptide includes a TRAF-interacting domain selected from the group consisting of: a) a LAP1 coiled coil domain; b) a LAP1 carboxy terminal domain extending

from amino acids 406-568 of SEQ ID NO:1; c) an EBI6 coiled coil domain; d) an EBI6 carboxy terminal domain extending from amino acids 259-416 of SEQ ID NO:2; e) a TRAF-interacting TNFR cytoplasmic domain; f) a TRAF metal binding domain.

28. The method or medicament of claim 26 in which the compound interacts with an oligomerizing TRAF domain.

29. The method or medicament of claim 28 in which the polypeptide includes a human TRAF coiled coil domain.

30. The method or medicament of claim 26 in which the polypeptide includes the sequence (amino acids 309-341 of SEQ ID NO:1):

L R N N E S K I L H L Q R V I D S
Q A E K L K E L D K E I R P F R.

31. The method of claim 23 or claim 25, or the medicament of claim 24, in which the compound is administered to a patient with undesired lymphocyte proliferation, manifest as an autoimmune disease, e.g., rheumatoid arthritis, Crone's disease, lupus, or to a patient characterized by drug induced immunosuppression.

III. Purified Polypeptides, Reagents and Methods For Making Them

32. A purified polypeptide capable of controlling TRAF-Mediated TNF/TNFR signal transduction when administered to a TRAF-encoding cell.

33. The polypeptide of claim 32 in which said polypeptide is capable of inhibiting interaction between

EBV LMP1 and a Tumor necrosis factor Receptor Associated Factor (TRAF) protein.

34. The polypeptide of claim 32 in which the polypeptide includes a LMP1-interacting TRAF domain.

35. The polypeptide of claim 34 in which the polypeptide includes a LMP1-interacting domain of a human TRAF protein.

36. The polypeptide of claim 35 in which the polypeptide includes the LMP1-interacting domain of LAP1.

37. The polypeptide of claim 36 in which the polypeptide includes a LMP1-binding domain within the LAP1 sequence between amino acids 345 and 568 of SEQ ID NO:1).

38. The polypeptide of claim 32 in which the polypeptide interacts with the following LMP1 sequence: G Q R H S D E H H H D D S L P H P Q Q A T D D S G H E S D S N S N E G R H H L L V S G A (SEQ ID NO:3).

39. The polypeptide of claim 32 in which the polypeptide interacts with a TRAF protein.

40. The polypeptide of claim 39 in which the polypeptide includes a LMP1 sequence that interacts with LAP1.

41. The polypeptide of claim 32 in which the polypeptide includes a domain selected from the group consisting of: a) a LAP1 coiled coil domain; b) a LAP1 carboxy terminal domain extending from amino acids 406-568 of SEQ ID NO:1; c) a LAP1 metal binding domain; d) an

EBI6 coiled coil domain; e) an EBI6 carboxy terminal domain extending from amino acids 259-416 of SEQ ID NO:2; and f) a TRAF-interacting cytoplasmic domain of a TNFR.

42. The polypeptide of claim 32 in which the polypeptide interacts with a LMP1-binding domain within the LAP1 sequence between amino acids 345 and 568 of SEQ ID NO:1).

43. The polypeptide of claim 32 in which the polypeptide includes a TRAF oligomer-forming domain.

44. The polypeptide of claim 43 in which the polypeptide includes a human LAP oligomer-forming domain.

45. The polypeptide of claim 44 in which the polypeptide includes a human LAP1 oligomer-forming domain.

46. The polypeptide of claim 45 in which the polypeptide includes the following LAP1 sequence (amino acids 309-341 of SEQ ID NO:1):

L R N N E S K I L H L Q R V I D S
Q A E K L K E L D K E I R P F R

47. The polypeptide of claim 32 in which the polypeptide includes a TRAF protein-interacting TNRF cytoplasmic domain that includes the sequence: $TX_{1-4}EE/DX_{0-2}K$, where T, E, D, and K are standard single letter amino acid designations, X can be any amino acid, X_{0-4} and X_{0-2} indicate optionally, from 0-4 or 0-2 amino acid
residues, respectiveliy, and E/D indicates a single amino acid residue that is either E or D.

48. A medicament for controlling TRAF-Mediated TNF/TNFR signal transduction when administered to a TRAF-encoding cell, said medicament comprising the polypeptide of claims 32-48.

49. Purified recombinant nucleic acid encoding the polypeptide of claim 32.

50. The recombinant nucleic acid of claim 49 in which the recombinant nucleic acid further comprises regulatory DNA positioned to transcribe the polypeptide encoding DNA.

51. A cell comprising the recombinant nucleic acid of claim 50.

52. A medicament for controlling TRAF-Mediated TNF/TNFR signal transduction when administered to a TRAF-encoding cell, said medicament comprising the purified nucleic acid of claim 50 or the cell of claim 51.

IV. Screening Techniques

53. A method of making the purified polypeptide of claim 32, comprising

culturing a cell containing recombinant nucleic acid encoding the polypeptide, the recombinant nucleic acid further comprising regulatory DNA positioned to transcribe the polypeptide-encoding nucleic acid in the cell, and

recovering the purified polypeptide from the cell or the culture medium.

54. A method of *in vitro* screening for substances that inhibit Tumor necrosis factor Receptor Associated Factor (TRAF) protein-related signal transduction, by:

a. providing a compound that includes a TRAF interacting domain; and

b. combining the compound with a candidate inhibitor;

c. determining whether the candidate inhibitor binds the compound.

55. The method of claim 54 in which the TRAF interacting domain is a) a LMP1 domain that includes the sequence G Q R H S D E H H H D D S L P H P Q Q A T D D S G H E S D S N S N E G R H H L L V S G A (SEQ ID NO:3); b) a LMP1-interacting LAP1 domain contained within amino acids 345-586 of SEQ ID NO:1; c) a TRAF-interacting TRAF domain; f) a TNFR-interacting TRAF domain; e) a TRAF-interacting TNFR cytoplasmic domain; or f) a TRAF metal binding domain.

56. A method of *in vitro* screening for substances that inhibit interaction of LMP1 with a Tumor necrosis factor Receptor Associated Factor (TRAF) protein, by:

a. providing a first binding partner and a second binding partner in a system that permits the binding partners to interact with each other, the first binding partner having a TRAF protein-interacting LMP1 domain, the second binding partner interacting with the LMP1 domain of the first binding partner; and

b. providing a candidate compound in the system;

c. determining whether the candidate compound inhibits interaction of the first binding partner and the second binding partner in the system.

57. The method of claim 56 in which the first binding partner's TRAF-interacting domain is a LMP1 domain that includes the sequence G Q R H S D E H H H D D

S L P H P Q Q A T D D S G H E S D S N S N E G R H H L L V S G A (SEQ ID NO:3).

58. The method of claim 56 in which the second binding partner's LMP1-interacting domain is a LAP1 domain contained within amino acies 345-586 of SEQ ID NO:1.

59. The method of claim 56 in which the first binding partner is LMP1 and the second binding partner is LAP1.

60. The method of claim 56 in which one of the binding partners is immobilized on a solid phase, and the other binding partner is provided in a liquid contacting the solid phase.

61. The method of claim 56 wherein the other binding partner includes a label to permit its detection.

62. The method of claim 56 in which the solid phase is part of an electrical circuit, and binding by the other partner is detected by measuring electrical characteristics of the circuit.

63. A method of *in vivo* screening for substances that inhibit interaction of LMP1 with a Tumor necrosis factor Receptor Associated Factor (TRAF) protein, by:

providing cells that express a TRAF and LMP1, interaction between the TRAF and LMP1 resulting in a detectable phenotypic trait.

64. The method of claim 63 in which interaction between the TRAF and the TRAF-interacting molecule is determined by detecting a phenotype of EBV infection.

65. A method of *in vitro* screening for substances that inhibit interaction of a Tumor necrosis factor Receptor Associated Factor (TRAF) protein with a member of the tumor necrosis factor receptor family, by:

a. providing a first binding partner and a second binding partner in a system that permits the binding partners to interact with each other, the first binding partner having a TRAF protein-interacting TNFR domain, the second binding partner comprising a TRAF protein domain that interacts with the TNFR domain of the first binding partner; and

b. providing a candidate compound in the system;

c. determining whether the candidate compound inhibits interaction of the first binding partner and the second binding partner in the system.

66. The method of claim 65 in which the TRAF protein-interacting domain is a TNRF cytoplasmic domain that includes the sequence: $TX_{1-4}EE/DX_{0-2}K$, where T, E, D, and K are standard single-letter amino acid designations, X can be any amino acid, X_{0-4} and X_{0-2} indicate optionally, from 0-4 or 0-2 amino acid residues, respectively, and E/D indicates a single amino acid residue that is either E or D.

67. The method of claim 65 in which the TRAF protein domain is a TNFR-interacting domain contained within the LAP1 amino acid sequence 345-586 of SEQ ID NO:1 or the EBI6 amino acid sequence 259-416 of SEQ ID NO:2.

68. The method of claim 65 in which the TNFR protein domain is a TRAF-interacting sequence within the cytoplasmic domain of a TNFR family member.

69. The method of claim 68 in which the TNFR family member is selected from the group consisting of p80, CD40, lymphotoxin-β, p60, and Fas.

70. Antibodies that specifically bind to a LAP1 or EBI6 immunodeterminant.

71. Antibodies raised by challenge with an antigen that contains a LAP1 or EBI6 immunodeterminant.

72. Purified human Epstein-Barr virus Induced Protein-6 (EBI6).

73. A purified human LAP.

74. Purified human LAP1.

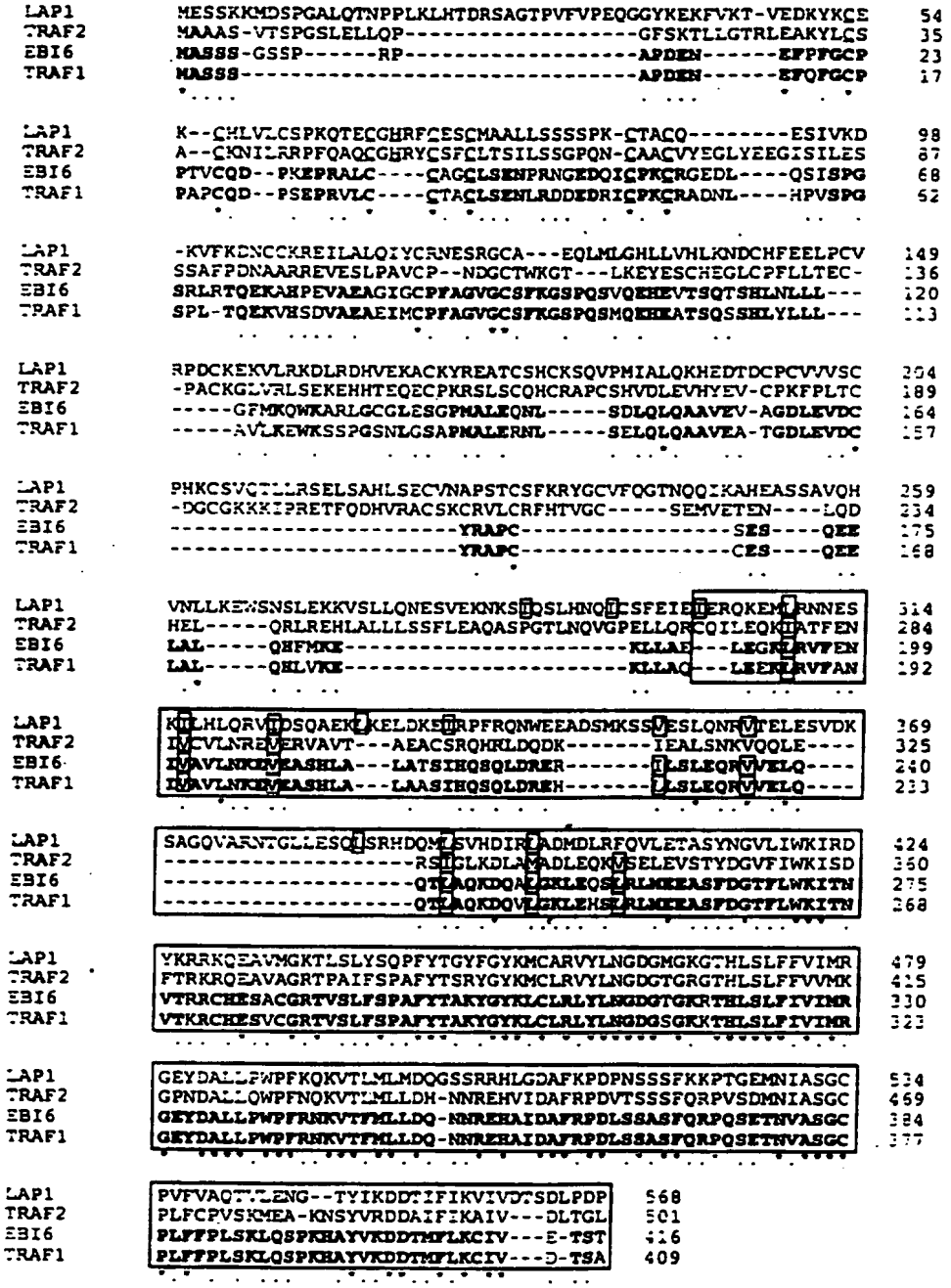
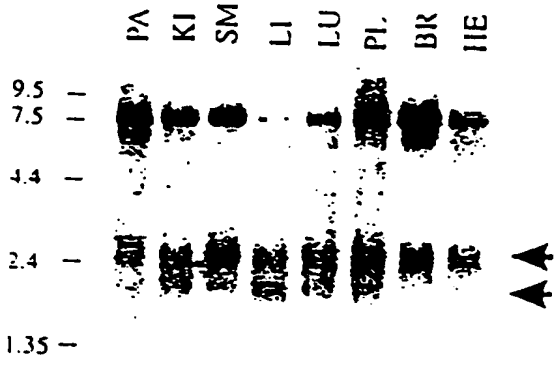


Figure 1

Fig.2

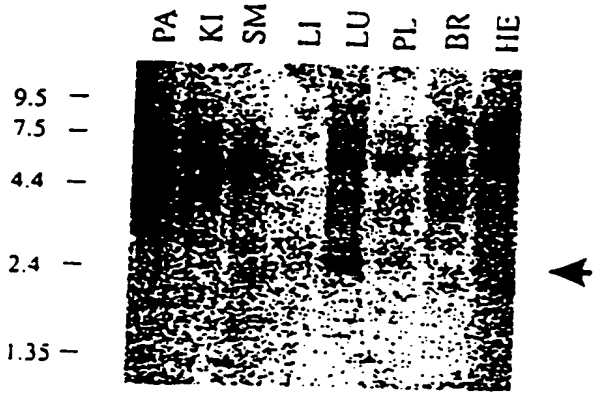
A



LAP1

Fig.2

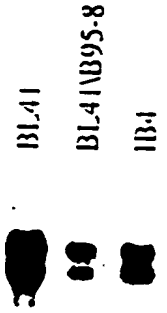
B



EBI6

Fig.2

C

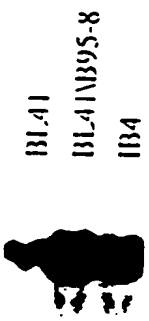


LAP1

ACTIN

Fig.2

D



EBI6

ACTIN

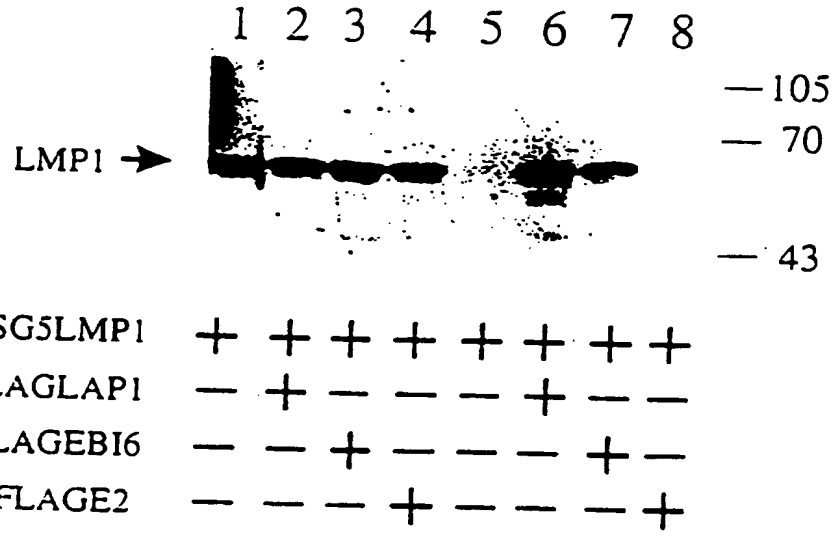


Figure 3

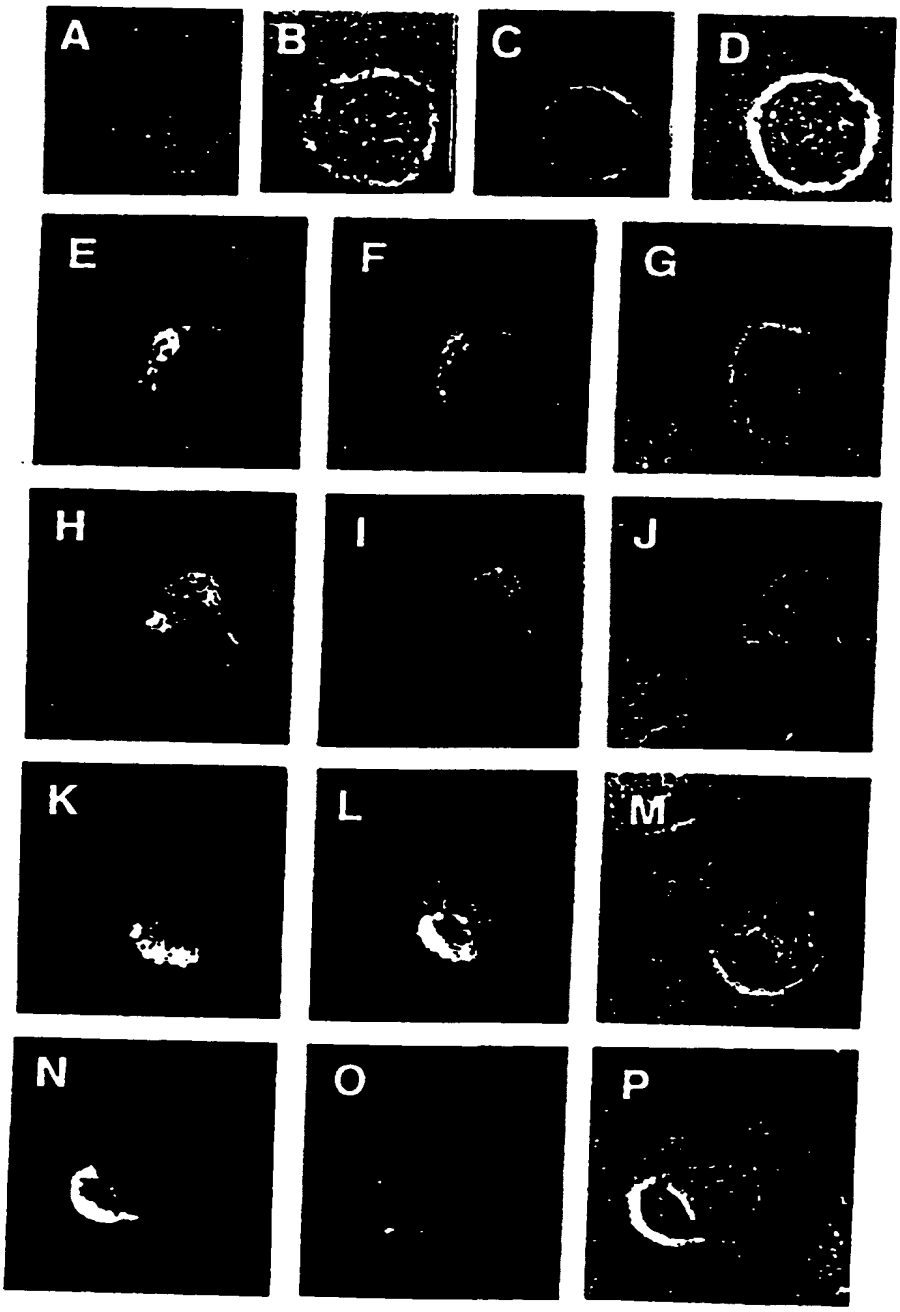


Figure 4

Fig.5

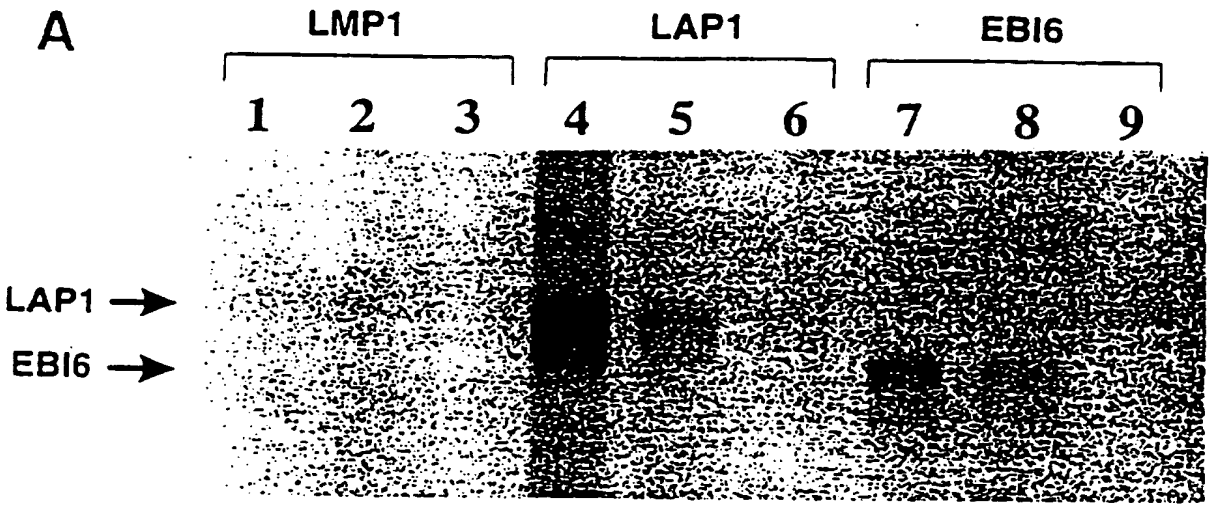

A
LMP1
LAP1
EBI6
1 2 3 4 5 6 7 8 9
LAP1
EBI6

Fig.5

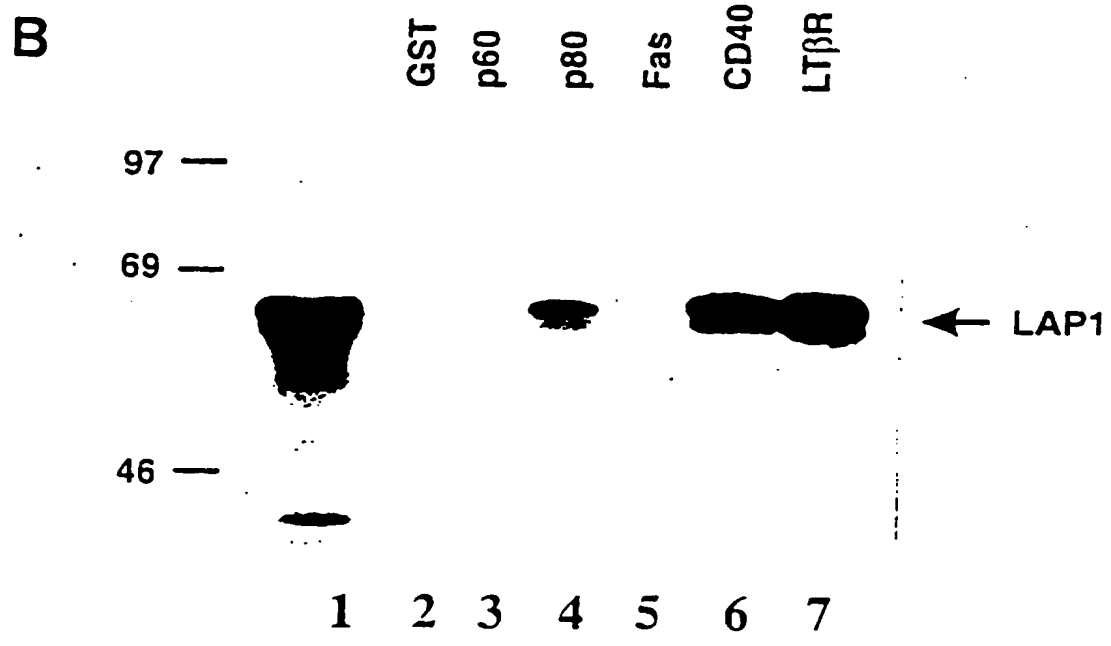

B
GST
p60
p80
Fas
CD40
LTβR
97
69
46
LAP1
1 2 3 4 5 6 7

Fig.5

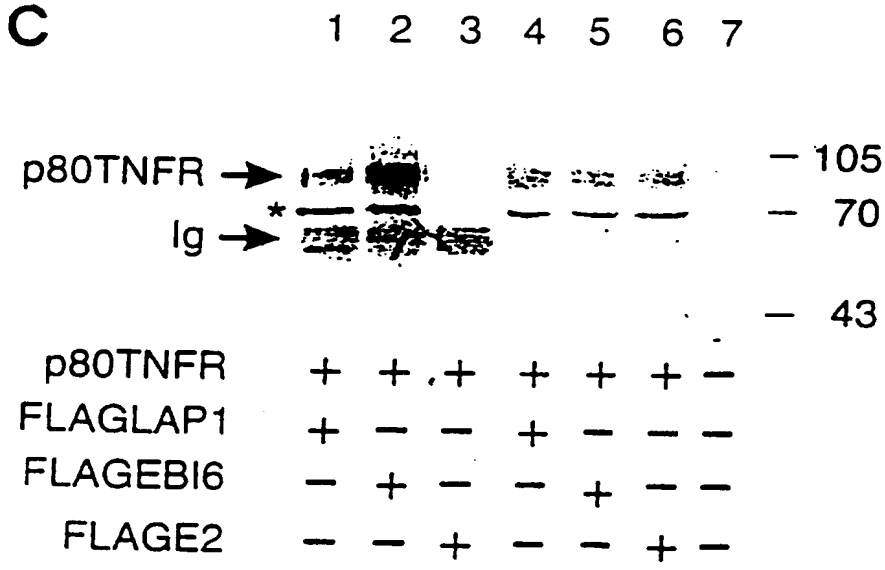

C
1 2 3 4 5 6 7
p80TNFR
Ig
*
105
70
43
p80TNFR + + + + + + −
FLAGLAP1 + − − + − − −
FLAGEBI6 − + − − + − −
FLAGE2 − − + − − + −

Fig.6

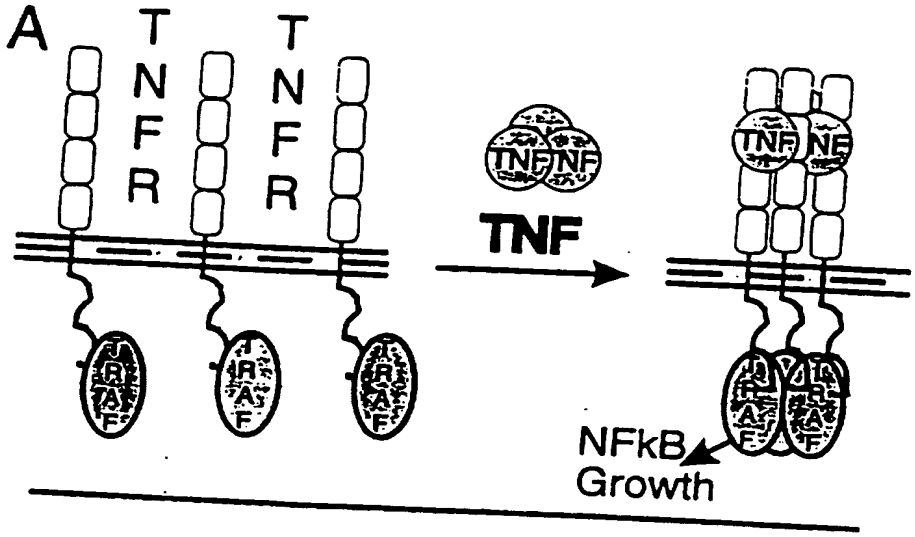
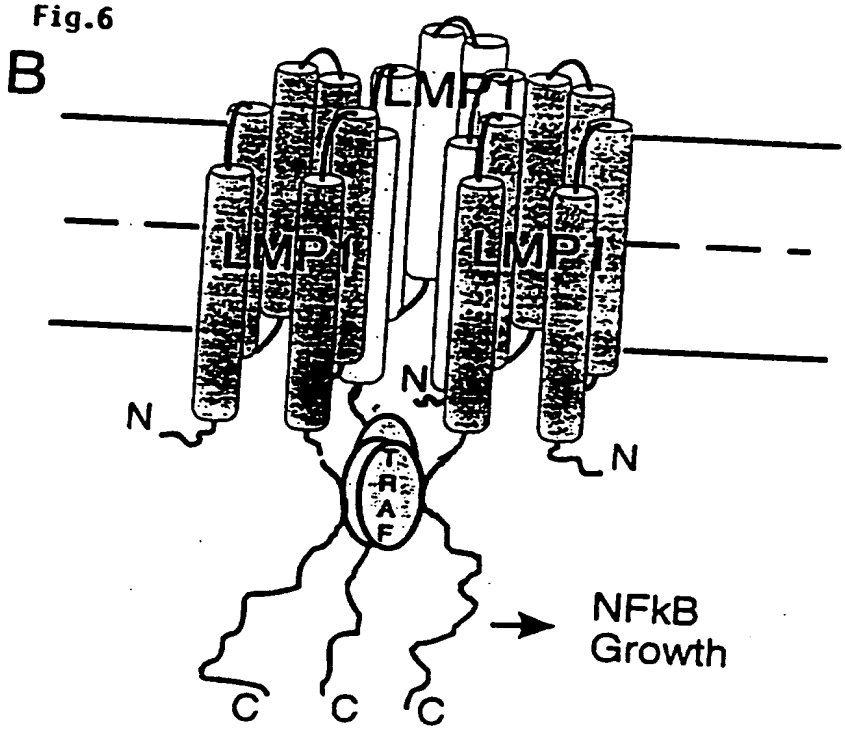

A
TNFR
TNFR
TRAF
TRAF
TRAF
TNF
TNF
TNF
TNF
TRAF
TRAF
TRAF
NFkB
Growth

Fig.6


B
LMP1
LMP1
LMP1
N
N
N
TRAF
NFkB
Growth
C
C
C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16980

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/02; C07K 4/12; C12P 21/02
US CL : 514/12; 530/300, 324, 350; 536/23.5; 435/69.1, 172.1, 252.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12; 530/300, 324, 350; 536/23.5; 435/69.1, 172.1, 252.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Searched Sequences SEQ ID NO: 1-3 on CAS ONLINE
Seacrhed APS, MEDLINE, DIALOG
search terms: TRAF, TNFR, EBV, LMP1, LAP1, method of treatment

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X --- P, Y	Cell, Volume 80, issued 10 February 1995, Mosialos et al, "The Epstein-Barr Virus Transforming Protein LMP1 Engages Signalling Proteins for the Tumor Necrosis Factor Receptor Family", pages 389-399, especially pages 390 and 392-395.	72-74 --- 1-51 and 53
X, P --- Y, P	Journal of Virology, Volume 69, No. 2, issued February 1995, Kaye et al, "The Epstein-Barr Virus LPM1 Cytoplasmic Carboxy Terminus Is Essential for B-Lymphocyte Transformation; Fibroblast Cocultivation Complements a Critical Function within the Terminal 155 Residues", pages 675-683, especially pages 676 and 681-682.	72-74 --- 1-51 and 53

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
23 APRIL 1996

Date of mailing of the international search report
20 MAY 1996

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Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16980

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-51, 53 and 72-74 drawn to a medicament for treating Epstein-Barr Virus (EBV)-associated infection, cell growth, tumorogenesis, rheumatoid arthritis, Crone's disease or lupus by administering a polypeptide compound that inhibits TRAF/TNFR cell growth/death signal transduction; the process of making the medicament and the process of using the medicament.
Group II, claim(s) 52, drawn to a medicament for controlling TRAF-Mediated TNF/TNFR signal transduction, said medicament comprising purified nucleic acid or a cell expressing purified nucleic acid.
Group III, claim(s) 54-62, drawn to a method for in vitro screening for substances that inhibit Tumor necrosis factor Receptor Associated Factor (TRAF) protein related signal transduction.
Group IV, claim(s) 63-64, drawn to a method for in vivo screening for substances that inhibit the interaction of LMP1 with a Tumor necrosis factor Receptor Associated Factor (TRAF) protein.
Group V, claim(s) 65-69, drawn to a method for in vitro screening for substances that inhibit the interaction of Tumor necrosis factor Receptor Associated (TRAF) protein with a member of the tumor necrosis factor receptor family.
Group VI, claim(s) 70-71, drawn to antibodies that specifically bind to a LAP1 or EBI6 immunodeterminant.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
Group I recites the special technical feature of administering a polypeptide compound that inhibits TRAF/TNFR cell growth/death signal transduction that is not needed for the inventions of Groups II-VI.
Group II recites the special technical feature of administering purified nucleic acid or a cell expressing purified nucleic acid for controlling TRAF-Mediated TNF/TNRF signal transduction that is not needed for the inventions of Groups I and III-VI.
Group III recites the special technical feature of an in vitro screening method for substances that inhibit Tumor necrosis factor Receptor Associated Factor (TRAF) protein related signal transduction that is not needed for the inventions of Groups I-II and IV-VI.
Group IV recites the special technical feature of an in vivo screening method for substances that inhibit the interaction of LMP1 with a Tumor necrosis factor Receptor Associated Factor (TRAF) protein that is not needed for the inventions of Groups I-III and V-VI.
Group V recites the special technical feature of an in vitro screening method for substances that inhibit the interaction of Tumor necrosis factor Receptor Associated (TRAF) protein with a member of the tumor necrosis factor receptor family that is not needed for the inventions of Groups I-IV and VI.
Group VI recites the special technical feature of antibodies that specifically bind to a LAP1 or EBI6 immunodeterminant that are not needed for the inventions of Groups I-V.